Relations phylogénétiques chez les termites du genre Reticulitermes en Europe. Description d'une nouvelle espèce.

Paolo Uva

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Paolo UVA

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Directeur de thèse :
Dr. BAGNÈRES-URBANY Anne-Geneviève

Jury :

CARIOU Marie-Louise, Directeur de Recherche	CNRS, Gif-sur-Yvette
ROISIN Yves, Professeur	Université de Bruxelles (Belgique)
BAGNÈRES-URBANY Anne-Geneviève, CR1 CNRS	Université de Tours
CLÉMENT Jean-Luc, Professeur	CNRS, Paris / Université de Tours
ERRARD Christine, Maître de Conférence	Université de Tours
MARINI Mario, Professeur	Université de Bologne (Italie)

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Et *grazie* à ma famille pour tout son soutien, et qui continue à se poser la question "Est-ce vraiment nécessaire de partir pendant si longtemps et si loin pour étudier des organismes aussi petits ?".

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Avant-propos

En milieu naturel, les termites jouent un rôle important dans la dégradation de la cellulose dans la plupart des écosystèmes. Leur présence en milieu urbain pose en revanche de nombreux problèmes lorsqu'ils s'attaquent aux constructions. En Europe, les termites souterrains du genre *Reticulitermes* comptent en effet parmi les insectes nuisibles les plus importants d'un point de vue économique.

Les méthodes actuelles de lutte des colonies nécessitent la mise au point de stratégies ciblées pour leur contrôle et leur traitement qui doivent être adaptés en fonction de l'espèce en présence (modalités biologiques de reproduction, vie cryptique, modes de propagation naturelle ou dus à l'homme). Des techniques d'identification précise des espèces doivent ainsi être disponibles. Plusieurs caractères ont ainsi été étudiés et apportent des informations aussi bien sur la biologie des termites que sur les critères utilisés comme clefs de discrimination interspécifique.

Nous avons ainsi employé une approche multidisciplinaire pour l'étude du genre *Reticulitermes* en Europe, avec une attention particulière pour le nouveau phénotype *Reticulitermes sp. nov.* identifié en France en 1998. Le but principal était d'identifier son aire d'origine et de définir sa position systématique dans un contexte évolutif. Cette approche s'est basée sur l'analyse des hydrocarbures cuticulaires, des substances défensive des soldats, ainsi que sur l'emploi de marqueurs mitochondriaux et nucléaires intervenant dans l'étude des relations évolutives.

Structure de l'ouvrage

La thèse a été présentée sous forme d'articles scientifiques. On comprendra alors la répétition de certains aspects, notamment pour les méthodes analytiques.

La partie introductive de l'ouvrage aborde des concepts d'ordre général. Le but a été celui de porter un regard sur l'ensemble de l'œuvre, tout en laissant la place pour des approfondissements où cela se révélait nécessaire.

Dans la section Matériel et Méthodes, l'ensemble des techniques a été reprise pour donner une vision globale des méthodes employées. Cette partie a été intégrée avec de nombreux aspects qui n'ont pas été présentés dans les différents articles.

Les résultats obtenus ont été inclus dans la troisième section de l'ouvrage. Les travaux réalisés en collaboration avec J. Austin et A. Quintana y sont également

présentés. Les résultats partiels obtenus au cours de mes trois années de thèse ont fait l'objet de plusieurs communications à des congrès scientifiques au niveau national et international (inclus dans la partie "Communications scientifiques").

Annexe confidentielle de la thèse

En 1998, tandis que j'effectuais mon stage de DEA au sein du Laboratoire de Neurobiologie (CNRS-UPR 9024), une enquête a été entreprise par le laboratoire (S. Ziani étudiant en DEA, A.-G. Bagnères et J.-L. Clément) sur la demande de la commune de Domène (Isère) pour délimiter les zones infestées par les termites dans la ville. Au cours de cette étude, les analyses morphologiques et chimiques ont permis d'identifier ce phénotype comme nouveau pour la France.

Dans le cadre d'un contrat CNRS / Ville de Domène, la découverte du nouveau phénotype a fait l'objet d'une recherche fondamentale au cours de ma thèse, à savoir la connaissance de sa position systématique au sein du genre *Reticulitermes*, mais aussi l'objet d'une étude appliquée pour répondre aux besoins de la ville de Domène.

Précisons que pour des raisons de confidentialité liées au contrat avec la ville de Domène, les résultats concernant l'étude appliquée sont présentés dans l'*Annexe confidentielle* de cette thèse. Les données relatives à la partie fondamentale "Origine et position systématique de la nouvelle espèce" sont donc exposées dans le présent ouvrage.

Introduction

Les termites dont le nom vient du latin "*termes*" qui signifie "ver rongeur", appartiennent à l'ordre des Isoptères. Ce sont des insectes sociaux se nourrissant de toutes sortes de matériaux à base de cellulose.

Les termites souterrains appartenant au genre *Reticulitermes* vivent dans des sociétés durables composées de différentes castes. Les individus sexués sont fondamentalement de deux types: imaginaux (ou reproducteurs primaires) et néoténiques (ou reproducteurs secondaires). Les premiers (mâles et femelles formant les couples royaux) ont une livrée plus foncée, des yeux normaux et des ailes bien développées, qu'ils perdent cependant immédiatement après le vol d'essaimage. Les seconds ont un tégument peu pigmenté, des yeux petits ou rudimentaires et des ailes réduites ou totalement absentes. Ce sont des individus à l'état larvaire mais dont les gonades sont développées, ce qui leur permet de se reproduire au sein de la colonie, entre eux ou avec un des reproducteurs primaires. Ces reproducteurs dits "de remplacement" jouent donc un rôle important dans la dynamique de reproduction et d'expansion des colonies. Les ouvriers, quant à eux, sont les plus nombreux avec un répertoire d'activités très étendu : fourragement, construction de galeries, échanges trophallactiques de nourriture. Avec l'autre caste spécialisée des soldats, ils contribuent aussi à la défense de la colonie.

Le genre Reticulitermes *en Europe : une taxonomie en évolution*

L'ordre des Isoptères compte environ 2600 espèces dans le monde entier (Kambhampati et Eggleton, 2000). Le genre *Reticulitermes* Holmgren 1913, à distribution holarctique (figure 1), est représenté par 90 espèces, dont six en Europe.

La première espèce européenne de *Reticulitermes*, *Termes lucifugum*, a été décrite par Rossi (1792) à partir d'échantillons récoltés en Italie. Par la suite, toutes les populations de termites trouvées en Europe ont été attribuées à ce taxon. Une deuxième espèce a été ensuite décrite par Kollar (1837) en Autriche, sous le nom de *Reticulitermes flavipes*. Cette espèce a été ensuite découverte aux Etats-Unis où elle

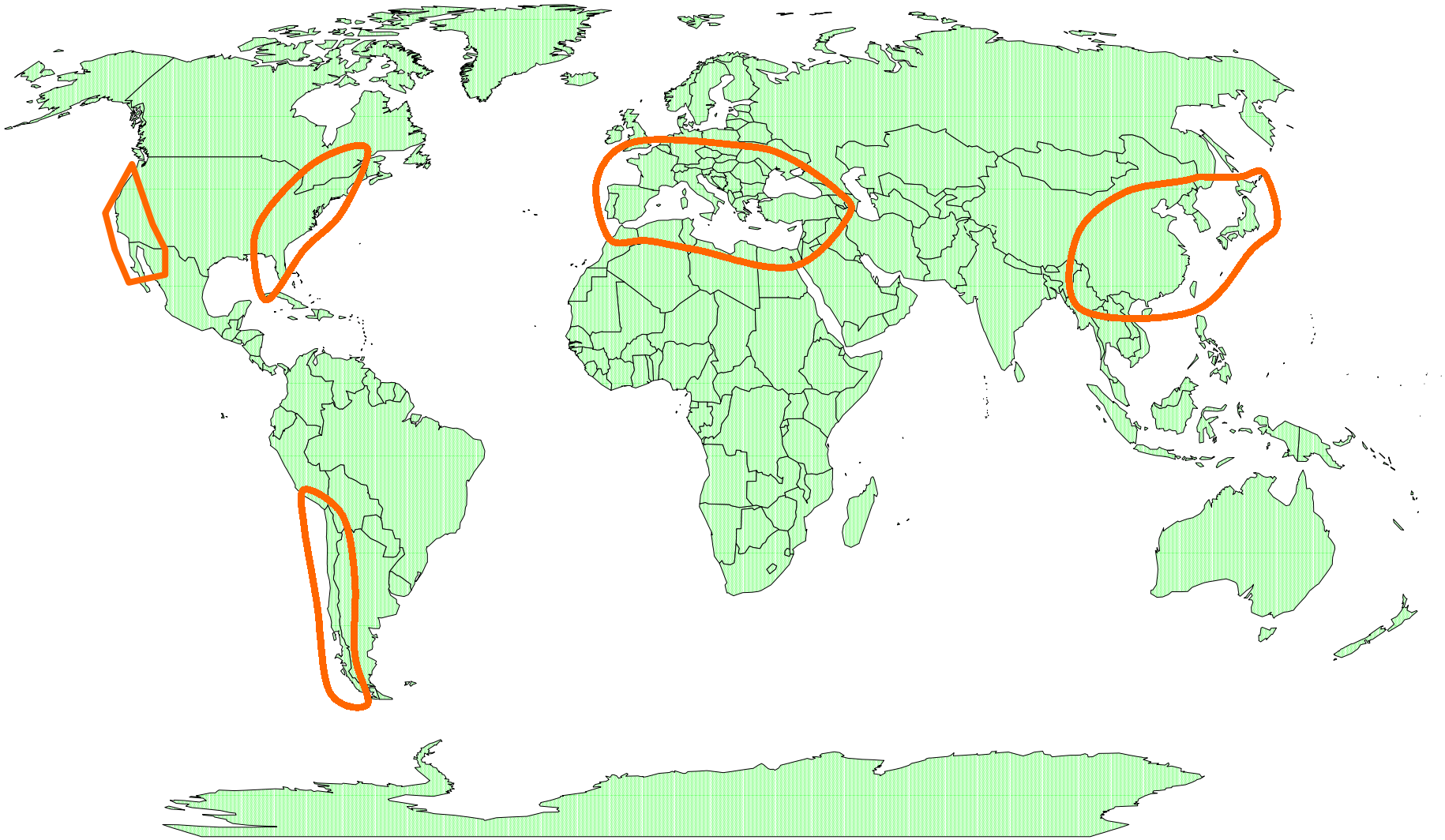


Figure 1. Aire de répartition des termites du genre *Reticulitermes* dans le monde

représente finalement l'espèce prédominante de *Reticulitermes* sur la côte Est (Weesner, 1970 ; Page et al., 2002).

En 1856, Lespes (1856) a signalé des termites dans la région de Saintonge (sud-ouest de la France) qui ne correspondaient pas à la détermination faite par Rossi. Feytaud (1924) leur a attribué par la suite le statut de sous-espèce *Reticulitermes lucifugus santonensis*. Ce nouveau taxon a par la suite fait l'objet de nombreux travaux : Jucci (1924) et même Feytaud (1925) sont les premiers à reporter une forte similarité morphologique avec l'espèce américaine *Reticulitermes flavipes* et ont proposé de les regrouper sous une même espèce, contrairement à ce qui sera affirmé par Lash (1952), Grassé (1954) et Clément (1978). Cette controverse continue encore actuellement avec la synonymie de ces deux espèces proposée par de nombreux auteurs (Bagnères et al., 1990 ; Jenkins et al., 2001 ; Vieau, 2001 ; Marini et Mantovani, 2002).

Ainsi, jusqu'aux années 1970, trois espèces de *Reticulitermes* sont connues en Europe : *Reticulitermes lucifugus*, distribuée du Portugal à l'Est de l'Europe, *R. flavipes* dans quelques villes d'Allemagne (Becker, 1970), et enfin *R. santonensis* en milieu naturel dans l'ouest de la France et dans plusieurs site urbains tels que Bordeaux et Nantes. Parmi ces trois taxa, seule *R. lucifugus* serait une espèce indigène appartenant à l'entomofaune européenne naturelle, les deux autres étant très probablement importées par l'homme.

Il faut attendre les années 1970-1980 pour que de nouvelles méthodes telles que la biométrie (Clément, 1978, 1979), la chimie (Parton et al., 1981) et la biochimie (Clément, 1981) permettent d'identifier plusieurs groupes de populations à l'intérieur du taxon *Reticulitermes lucifugus*. Dans un premier temps, ces populations ont eu le statut de sous-espèce en l'absence de preuves sur leur inter-stérilité naturelle (Clément, 1978). La présence de mécanismes d'isolement reproductif (géographiques et physiologiques) a par la suite amené à définir un certain nombre d'espèces morphologiquement identiques (*sibling species*) (recensées par Clément et al., 2001) (figure 2) :

- *R. lucifugus* (Rossi) en Italie et le sud-est de la France (Provence)
    - la sous-espèce *R. lucifugus corsicus* Clément en Corse (France) et en Sardaigne (Italie)

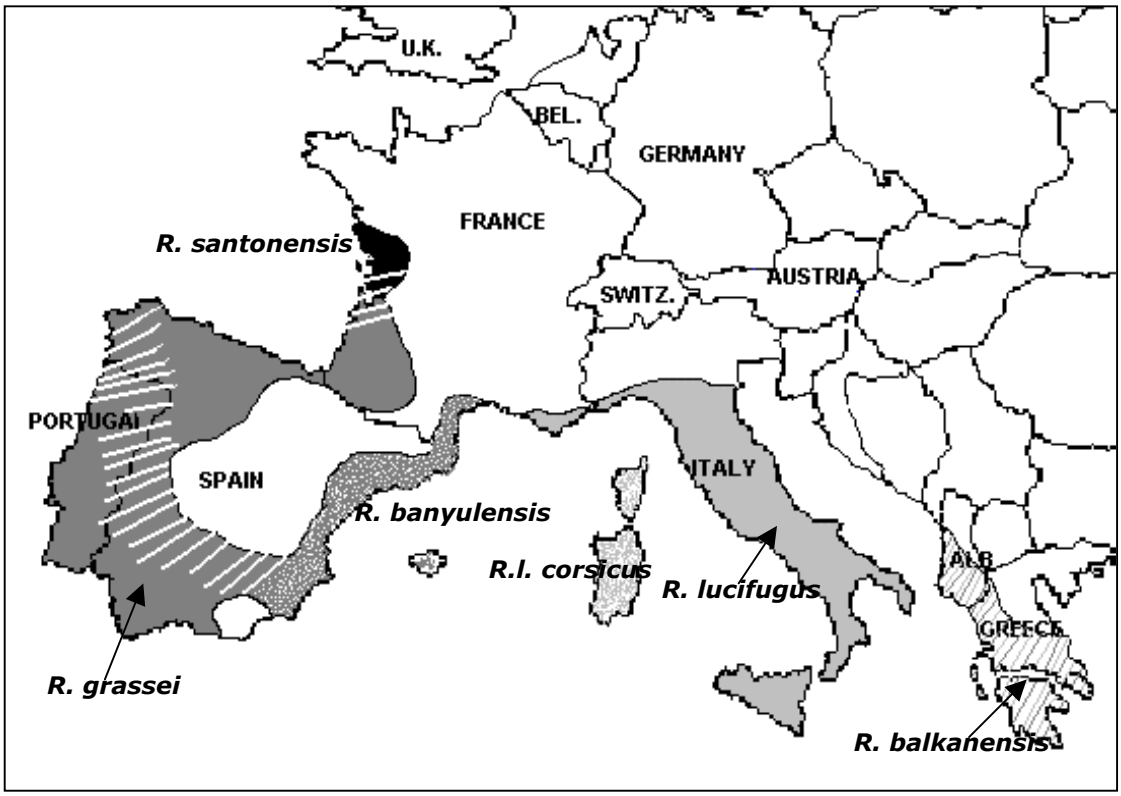


Figure 2. Aire de répartition des termites du genre *Reticulitermes* en milieu naturel (Clément et al., 2001).

- *R. grassei* Clément dans le sud-ouest de la France, le nord-ouest et le sud de l'Espagne et le Portugal
- *R. banyulensis* Clément dans le nord-est de l'Espagne, et le sud-est de la France (Languedoc-Roussillon)
- *R. balkanensis* Clément dans les Balkans.

En 1998 un nouveau phénotype a été identifié en milieu urbain par A.-G. Bagnères et retrouvé dans la même année dans une ville du Nord de l'Italie.

Systématique moléculaire du genre Reticulitermes

Durant ces dix dernières années, de nombreux travaux de phylogénie moléculaire sur le genre *Reticulitermes* ont permis d'établir les relations évolutives au sein de ce taxon (Jenkins et al., 2000, 2001 ; Marini et Mantovani, 2002), mais également de l'intégrer à d'autres niveaux taxonomiques supérieurs (Kambhampati, 1995; Lo et al., 2000; Eggleton, 2001 ; Maekawa et al., unpublished data).

Les Reticulitermes *en GenBank*

À ce jour, 172 séquences de *Reticulitermes* sont déposées dans GenBank (figure 3) : leur nombre n'a cessé d'augmenter depuis 1994, mais on observe une grande hétérogénéité dans leur distribution pour les espèces et les régions représentées (figure 4).

Mis à part l'isolement de marqueurs microsatellites (Vargo, 2000) et l'utilisation d'autres séquences nucléaires codant pour des cellulases (Watanabe et al., 1998 ; Tokuda et al., 1999), toutes les autres séquences dérivent de l'amplification de l'ADN mitochondrial.

Les gènes mitochondriaux les plus étudiés correspondent à ceux codant pour les cytochromes oxydases I et II (COI et COII), alors que les autres gènes codants sont moins utilisés, ce qui est en accord avec les observations de Caterino et al. (Caterino et al., 2000). De plus, plusieurs auteurs ont utilisé les mêmes gènes pour étudier les mêmes espèces de *Reticulitermes*. Il s'agit là d'un problème déjà souligné par Eggleton (2001) : l'échantillonnage requis pour l'étude phylogénétique n'est en effet pas souvent réalisé avec un effort conjoint au niveau international.

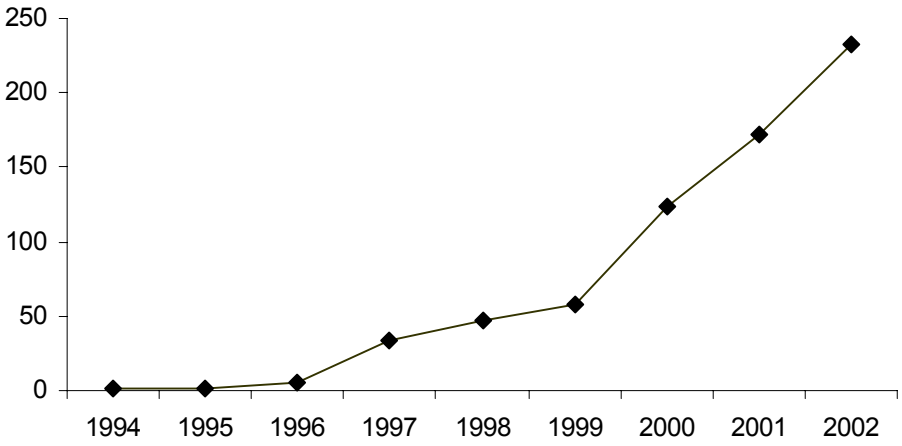


Figure 3. Nombre de séquences déposées dans GenBank pour le genre *Reticulitermes*.

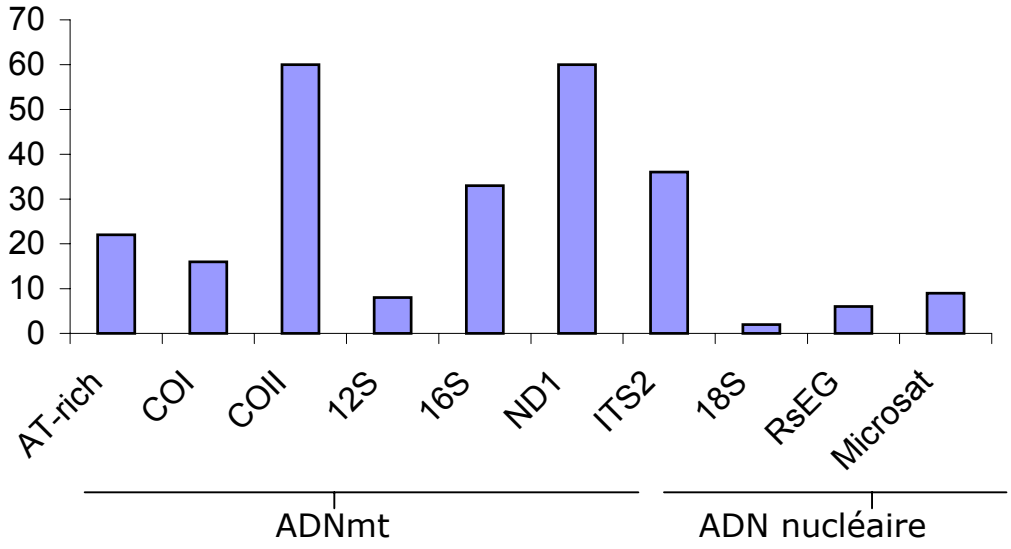


Figure 4. Nombre de séquences déposées dans GenBank classées par marqueur moléculaire.

En examinant les différentes aires géographiques échantillonnées, on observe une distribution assez homogène mais limitée des séquences : une à deux séquences pour chacune des onze espèces de *Reticulitermes* des régions Est-Paléarctique et Orientale (Ohkuma, unpublished data ; Xing et al., unpublished data).

Dans la région Néarctique, Jenkins et ses collaborateurs ont étudié la structure des populations de *Reticulitermes* (Jenkins et al., 1998, 1999), ce qui explique un nombre élevé de séquences déposées dans GenBank pour un même taxon. Dans cette région, certains taxa restent néanmoins exclus (*R. hesperus*, *R. tibialis* et *R. arenicola*).

Dans la région Ouest-Paléarctique, notamment dans le pourtour méditerranéen, la situation est assez inattendue : bien qu'il s'agisse d'une aire connue d'un point de vue faunistique où l'on peut supposer que quasiment toutes les espèces de termites ont été décrites (Eggleton, 1999), le nombre de séquences par espèce est loin d'être homogène. En outre, deux espèces n'y sont pas représentées (*R. banyulensis* et *R. balkanensis*).

Dans la partie Est du bassin méditerranéen aucun travail de phylogénie moléculaire n'a encore été réalisé.

Le concept d'espèce – Le cas de R. sp. nov.

Après la découverte d'un nouveau phénotype de *Reticulitermes* en France en 1998, nous avons été amené à considérer la signification évolutive des différences morphologiques et chimiques observées. Très rapidement, nous nous sommes demandé s'il s'agissait d'une nouvelle espèce. Pour cela, nous devions définir le cadre dans lequel le concept d'espèce allait être employé dans cette étude.

Actuellement, de nombreuses définitions du concept d'espèce ont été proposées (Avise, 1994 ; Mayden, 1997 ; Hey, 2001). À titre d'exemples, nous pouvons dresser une liste non exhaustive des définitions les plus connues :

- Agamospecies Concept
- Biological Species Concept
- Cladistic Species Concept
- Cohesion Species Concept
- Composite Species Concept
- Ecological Species Concept
- Evolutionary Significant Unit
- Evolutionary Species Concept
- Genealogical Concordance Concept

- Genetic Species Concept
- Genotypic Cluster Concept
- Hennigian Species Concept
- Internodal Species Concept
- Morphological Species Concept
- Non-dimensional Species Concept
- Phenetic Species Concept
- Phylogenetic Species Concept (Diagnosable Version)
- Phylogenetic Species Concept (Monophyly Version)
- Phylogenetic Species Concept (Diagnosable and Monophyly Version)
- Polythetic Species Concept
- Recognition Species Concept
- Reproductive Competition Concept
- Successional Species Concept
- Taxonomic Species Concept

Certaines de ces définitions sont des variations du concept biologique de l'espèce (voir paragraphe suivant) basées sur les caractéristiques des populations. D'autres considèrent les espèces comme des groupes d'organismes avec des caractéristiques similaires.

Parmi les concepts d'espèce définis sur l'existence de populations, on retrouve les concepts de *cohésion* (présence de facteurs génétiques et écologiques qui créent une communauté reproductive cohésive), d'*espèce cladistique* (les populations locales et les sous-espèces sont élevées au rang d'espèce), d'*espèce évolutive* (lignées d'espèces) et le concept de *reconnaissance* (partage d'un même mécanisme de reconnaissance pour l'accouplement).

Parmi les concepts d'espèces qui se basent sur les similarités entre organismes, on peut citer les plus connus : l'*espèce phénétique* (plusieurs caractères sont utilisés pour définir les taxa), *morphologique* (similarité morphologique), *génétique* (gènes similaires) et *physiologique* (capacité physiologique des individus à se reproduire entre eux) (Ghiselin, 2001).

Notons que le concept de *cohésion* est le seul pour lequel un test statistique a été proposé (Templeton, 2001), les autres définitions étant dépourvues d'hypothèse à tester de façon rigoureuse.

Le concept d'espèce biologique (*Biological Species Concept* ou BSC) est le plus utilisé aujourd'hui. Initialement proposé par Mayr (1963), il considère les espèces

comme des systèmes de populations naturelles isolées reproductivement. Les individus d'une même espèce peuvent donc se reproduire entre eux directement ou indirectement, ce qui crée un flux génique au sein des populations. Il faut noter qu'il s'agit ici d'un isolement reproductif entre les espèces à l'état naturel, et non d'une incapacité à se reproduire (ce dernier point est à la base du *Physiological Species Concept*). En d'autres termes, les croisements réalisés en laboratoire ne rentrent pas dans le cadre du BSC (Ghiselin, 2001).

Un des problèmes majeurs du BSC, également valable pour d'autres concepts d'espèces, est de vouloir définir le processus graduel de spéciation avec une terminologie discrète (classification en espèces). Cette terminologie est néanmoins nécessaire pour "catégoriser" le vivant : on parle de l'espèce comme taxon, qui est le résultat conjoint de l'Évolution et de la tendance de l'homme à classer des patterns communs (Hey, 2001).

Pour s'affranchir de ces contraintes où l'espèce équivaut à un taxon, on s'intéresse aux mécanismes évolutifs pour désigner l'espèce comme un groupe évolutif. Celui-ci est considérée comme une entité dynamique séparée des autres par ses tendances évolutives (Simpson, 1951). D'un point de vue opérationnel, l'utilisation de cette définition identifier les groupes évolutifs d'une manière univoque reste encore difficile à réaliser en raison de son caractère assez vague.

La disponibilité de nouvelles techniques d'analyse, notamment l'utilisation de la biologie moléculaire, a permis à Cracraft (1983) de proposer un nouveau concept d'espèce : *espèce phylogénétique* (*Phylogenetic Species Concept* ou PSC). Il définit une espèce comme un groupe monophylétique composé d'individus avec un pattern commun d'ancêtres et de descendants. Néanmoins un problème se pose quant à l'établissement de la monophylie d'un groupe, puisqu'à chaque marqueur correspond un niveau de résolution différent. Dans ce cas les différences observées en dessous d'un certain seuil devraient être ignorées, mais le choix du seuil reste sûrement discutable (Avise, 1994 ; Avise et Walker, 1999, 2000 ; Hendry et al., 2000).

A la recherche d'un lien entre le BSC et PSC, Avise et Ball (1990) ont proposé le principe de *concordance généalogique* (GCC). Des regroupements phylogénétiques obtenus à partir de caractères indépendants peuvent différer entre eux en l'absence de forces évolutives unidirectionnelles. L'isolement reproductif est une force qui est censée générer des généalogies concordantes (le principe de BSC). De génération en

génération, les populations reproductivement isolées évoluent en groupes phylogénétiquement distincts. Même les barrières extrinsèques à la reproduction (isolement géographique) peuvent amener à une partition phylogénétique de populations, alors qu'elles seraient considérées comme appartenant à une même espèce selon le principe du BSC. Pour résoudre ce problème, les auteurs suggèrent de garder la classification d'espèce pour des populations reproductivement isolées par des facteurs intrinsèques ; les populations phylogénétiquement distinctes mais isolées par des barrières géographiques seraient, elles, considérées comme des sous-espèces.

Dans ce contexte de concordance généalogique, il était nécessaire de s'intéresser à des caractères indépendants dont la base génétique allait nous permettre d'établir des relations phylogénétiques.

Outils pour l'identification des espèces

Plusieurs techniques d'analyses peuvent être employées pour l'identification des espèces. Cette partie décrit plus en détail les méthodes qui ont été utilisées au cours de ma thèse : l'analyse des hydrocarbures cuticulaires, l'identification des substances défensives des soldats et l'emploi de marqueurs génétiques.

Les hydrocarbures cuticulaires

Structure et fonctions de la cuticule

La cuticule constitue une acquisition fondamentale dans l'évolution des arthropodes. Agissant comme une barrière entre l'animal et le milieu, elle a permis à de nombreux groupes de s'affranchir du milieu aquatique grâce à la limitation de la perte d'eau. Recouvrant la surface du corps, elle est composée d'une couche supérieure fine, l'épicuticule, et d'une couche inférieure plus épaisse, la procuticule (exocuticule et endocuticule) (figure 5). L'épicuticule, riche en lipides, est à la base de la rétention hydrique de l'organisme. La procuticule, composée essentiellement de chitine, assure l'intégrité structurale de la cuticule (Wigglesworth, 1973 ; Hadley, 1986 ; Nation, 2002).

L'épicuticule formée d'une cire est constituée majoritairement d'hydrocarbures cuticulaires qui sont des chaînes hydrocarbonées saturées (*n*-alcanes : alcanes avec chaînes linéaires saturées ; mono-, di-, tri-méthylalcane : 1, 2 ou 3 groupes

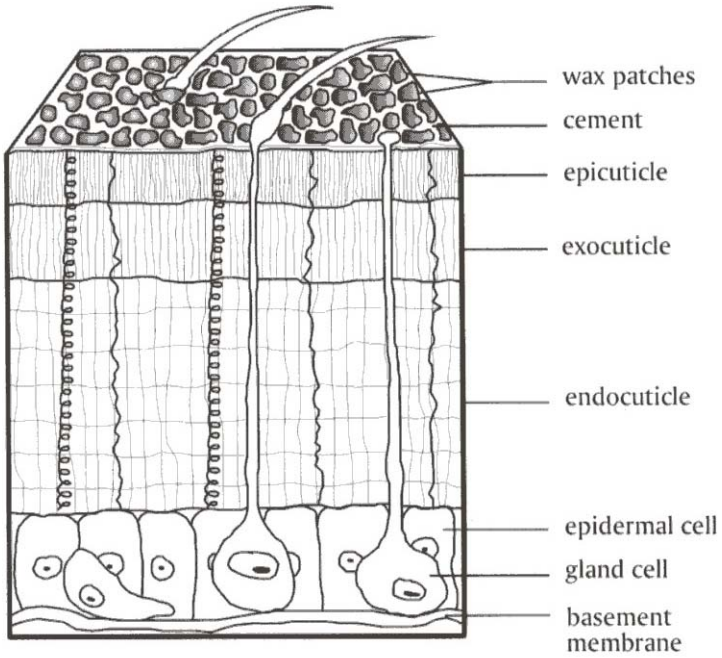


Figure 5. Structure de la cuticule chez les insectes (d'après Nation, 2002).

méthyle -CH_3) ou insaturées (monoènes, diènes, triènes : 1, 2 ou 3 doubles liaisons). Ils sont synthétisés par l'organisme à l'intérieur des oenocytes, cellules situées en proximité de l'épiderme (Lockey, 1988), et par la suite transportés dans l'hémolymphe et jusqu'à l'épiderme par des protéines de transport, les lipophorines. De l'épiderme, ils sont reversés sur la surface externe de la cuticule (Katase et Chino, 1982, 1984).

Variations intra- et inter-coloniales

Au sein d'une même espèce, les variations quantitatives que l'on observe entre les colonies d'insectes jouent un rôle clef dans les mécanismes de reconnaissance coloniale, et ont fait l'objet de nombreux travaux (**Hyménoptères sociaux** : Bonavita-Cougourdan et al., 1987, 1990 ; Brill et Bertsch, 1990 ; Brill et al., 1985, 1986 ; Butts et al., 1993 ; Dahbi et al., 1996 ; Henderson et al., 1990 ; Lenoir, 2002 ; Lenoir et al., 1988, 1999 ; Lorenzi et al., 1997 ; Nowbahari et al., 1990 ; Provost et al., 1994 ; Soroker et al., 1995 ; Vander Meer et al., 1989 ; **Isoptères** : Bagine et al., 1994 ; Bagnères et al., 1988, 1990 ; Blomquist et al., 1979 ; Brown et al., 1996 ; Howard et al., 1982 ; Su et Haverty, 1991 ; Takahashi et Gassa, 1995).

Le rôle joué par les hydrocarbures cuticulaires dans les mécanismes de reconnaissance est particulièrement évident dans le cas des insectes parasites qui peuvent pénétrer à l'intérieur d'une colonie en mimant la composition des hydrocarbures de leur espèce hôte (*Polistes atrimandibularis*, Bagnères et al., 1996, Lorenzi et al., 1996 ; *Polyergus rufescens*, Bonavita-Cougourdan et al., 1996, 1997 ; *Harpagoxenus sublaevis*, Kaib, 1993). La plasticité de cette signature chimique a également été observée dans des colonies mixtes expérimentales (Vienne et al., 1995 ; Bagnères et al., 1991 ; Vauchot et al., 1996, 1997, 1998).

Les hydrocarbures cuticulaires varient également qualitativement d'une espèce à l'autre en fonction du système enzymatique de synthèse et de régulation. Ces enzymes n'étant que le reflet des génotypes, l'étude de la composition des hydrocarbures fournit donc de façon indirecte des informations d'ordre génétique (Takahashi et al., 2001). De part leur caractéristique spécifique, les hydrocarbures cuticulaires peuvent être employés avec succès pour différencier des espèces.

Chaque espèce possède en effet un mélange d'hydrocarbures cuticulaires qui constitue une véritable signature chimique. Dans le cas des termites *Reticulitermes*, ce

mélange comprend 20 et 50 hydrocarbures cuticulaires. Deux individus appartenant à la même espèce peuvent ainsi être identifiés en comparant leurs profils cuticulaires.

Carlson et Service (Carlson et Service, 1980) ont été parmi les premiers à différencier deux espèces morphologiquement proches de Diptères par l'analyse des hydrocarbures cuticulaires. Les études qui ont suivi ont validé l'emploi des hydrocarbures en systématique dans plusieurs taxa (Diptères : Milligan et al., 1986 ; Hymenoptères : Nowbahari et al., 1990 ; Isoptères : Bagine et al., 1994 ; Brown et al., 1996 ; Haverty et al., 1990, 1996, 1997 ; Watson et al., 1989, et plus particulièrement les *Reticulitermes* : Clément et al., 1985 ; Bagnères, 1989 ; Bagnères et al., 1988, 1990, 1991).

Le mélange des hydrocarbures cuticulaires constitue donc un signal complexe apportant des informations sur l'espèce, la colonie, la caste d'appartenance, le stade de développement, etc.

Les substances défensives des soldats

Chez la plupart des espèces de termites, on observe la présence d'une caste spécialisée dans la défense de la colonie : les soldats des familles Rhinotermitidae (incluant le genre *Reticulitermes*) et Termitidae sont pourvus d'une glande frontale contenant une sécrétion qu'ils peuvent expulser à travers un pore frontal (Grassé, 1982). Ces sécrétions sont employées pour défendre la colonie des prédateurs (Quennedey et Deligne, 1975 ; Prestwich, 1979).

Bagnères et al. (1990) et Nelson et al. (2001) ont analysé les sécrétions de la glande frontale des *Reticulitermes* nord-américains et ont montré une variation de leur composition en fonction de l'espèce et de la localisation géographique. Tout comme les patterns observés à partir de l'analyse des hydrocarbures cuticulaires, ces variations ont été employées avec succès comme indicateurs taxonomiques (Haverty et al., 1999 ; Nelson et al., 2001). Des différences spécifiques ont également été observées chez les *Reticulitermes* européens (Clément et al., 2001).

Les marqueurs génétiques

La découverte de la réaction de polymérisation en chaîne (Mullis et al., 1986) est une étape fondamentale dans l'avancé de la biologie moléculaire qui a révolutionné les

domaines de la systématique et de la phylogénie. Les informations contenues dans les séquences nucléotidiques étaient enfin disponibles pour la reconstruction des relations évolutives entre taxa à différents niveaux de divergence.

Le génome mitochondrial

Parmi la panoplie de marqueurs génétiques disponibles, l'ADN mitochondrial a une place importante dans les analyses phylogéniques car il répond, en grande partie, aux caractéristiques du "système idéal" (Avise et al., 1987 ; Harrison, 1989 ; Moritz, 1994) :

- distribution ubiquitaire, pour une comparaison d'homologie pour une ample variété d'organismes
- utilisation simple (présence d'amorces universelles pour l'amplification par PCR)
- structure relativement simple (absence d'intron)
- transmission sans recombinaison et absence de réarrangements génétiques, mais voir également (Wallis, 1999 ; Hey, 2000) pour la recombinaison d'ADN mitochondrial (Zhang et Hewitt, 1996) et la présence de pseudogènes mitochondriaux pouvant introduire des erreurs dans les analyses phylogénétiques
- taux d'évolution suffisamment rapide pour que l'apparition de nouveaux caractères soit comprise dans la durée de vie de l'espèce.

À ces propriétés il faut également rajouter l'hérédité de type maternelle, permettant de distinguer et de suivre séparément le flux génique maternel du flux paternel, ainsi que la transmission clonale du génome mitochondrial sous forme d'un gène unique.

Le taille du génome varie entre 14kb et 39kb. Son contenu est assez uniforme : la majorité des taxa possède deux gènes codant pour l'ARN ribosomal, 22 gènes pour l'ARN de transfert et 13 gènes codant pour des protéines impliquées dans la chaîne de transport des électrons et la synthèse de l'ATP. À part les ARNt, l'ordre des gènes est conservé pour les vertébrés et les insectes, mais des variations ont été observées pour les autres phyla (Fukuhara, 1983 ; Moritz et al., 1987 ; Wolstenholme, 1992 ; Curole et Kocher, 1999).

Pour mieux comprendre les avantages et les limites d'un tel système dans les études phylogénétiques, il faut également considérer les contraintes structurales et

fonctionnelles, auxquelles de tels gènes sont soumis. Ces contraintes se reflètent dans les différents taux d'évolution observés. Les gènes ribosomaux sont les plus conservés, à l'opposé de la région de contrôle (A+T rich chez les insectes, D-loop chez les vertébrés) qui est extrêmement variable en composition et en taille ; les gènes codant pour des protéines ont normalement une position intermédiaire (Moritz et al., 1987). Ainsi la présence de taux d'évolution unique pour chaque gène permet de répondre à la plupart des problèmes phylogénétiques : la région à étudier sera choisie en fonction de la séparation des taxa en question (Brown, 1981 ; Brown et al., 1979 ; Russo et al., 1996 ; Simon et al., 1994 ; Zardoya et Meyer, 1996).

Les études de systématique basées sur l'analyse des séquences d'ADN mitochondrial font l'hypothèse que la phylogénie obtenue représente l'histoire évolutive des espèces observées. Chaque nœud correspond ainsi à un événement de spéciation.

En d'autres termes, l'histoire évolutive des gènes est considérée comme représentative de l'histoire évolutive des organismes, mais il s'agit d'une approximation car la "phylogénie des gènes" n'est pas la "phylogénie des espèces" (Pamilo et Nei, 1988 ; Crozier, 1990 ; Excoffier, 1997 ; Nichols, 2001). Suite à un événement de spéciation, la population dérivée aura un échantillon de gènes de la population mère. Une phylogénie basée sur les gènes présents actuellement au sein d'une population pourra ainsi inclure des éléments de la population d'origine, et ceci pourra amener à une différence entre l'arbre des gènes et l'arbre des espèces.

Pour réduire cette discordance, ou mieux, pour augmenter la probabilité de la détecter, plusieurs loci devraient être étudiés (Pamilo et Nei, 1988). Les modalités de transmission du génome mitochondrial sont telles que le génome peut être considéré comme un seul gène, donc l'utilisation de plusieurs régions d'ADNmt n'apportera pas *a priori* d'informations complémentaires à la résolution de la discordance. Dans ce contexte l'utilisation de gènes nucléaires est nécessaire pour trouver une réponse (Crozier, 1990) et réduire la probabilité d'obtenir une topologie incorrecte. Mais en même temps, l'ADNmt a une probabilité plus importante que le génome nucléaire de rendre l'arbre réel en raison de la taille inférieure de la population effective (influence de la transmission maternelle).

Une solution envisageable dans les études visant à reconstruire des relations phylogénétiques entre espèces consiste à utiliser une taille suffisamment informative

du génome mitochondrial, choisie en fonction du taux d'évolution des séquences et de la distance entre les espèces (voir Martin et al., 1990, et Cummings et al., 1995, pour l'effet de la taille de la séquence sur la probabilité d'obtenir la topologie réelle). Mais l'étude d'espèces strictement apparentées nécessite également d'utiliser des gènes nucléaires, ce qui diminue la divergence entre la phylogénie des gènes et la phylogénie des espèces. Pour les taxa d'ordre supérieur cette divergence est négligeable.

Le génome nucléaire : les ITS (Internal Transcribed Spacer)

À la recherche d'un signal phylogénétique dans le génome nucléaire, de nombreux gènes ont été employés en systématique (une révision récente de la systématique moléculaire des arthropodes a été réalisée par Caterino et al. en 2000).

Parmi les gènes nucléaires, les gènes ribosomaux (ARNr) ont attiré tout particulièrement l'attention en raison de leur abondance dans le génome, et leur conséquente facilité d'amplification. Ils sont constitués par une mosaïque de régions variables et conservées. Les zones conservées sont utilisées pour la reconstruction des relations phylogénétiques à des niveaux taxonomiques d'ordre supérieur, tandis que les régions variables (ITS, Internal Transcribed Spacer), bien que présentant souvent des problèmes d'alignement, peuvent être employées à un niveau taxonomique inférieur. Les gènes ribosomaux constituent ainsi des marqueurs moléculaires avec un large spectre d'action pouvant aller des relations entre phyla, jusqu'aux populations (Hillis et Dixon, 1991 ; Stackebrandt, 2001).

En particulier, la comparaison des séquences des ITS est devenue un outil largement employé pour différencier des espèces strictement apparentées (par exemple chez les Cicindèles, Vogler et DeSalle, 1994). Flanquée par des séquences d'ARNr très conservées, cette région peut être facilement amplifiée grâce à des amorces universelles.

Les ITS sont impliqués dans la maturation du transcrit primaire, et sont ainsi soumis à des contraintes structurales qui influencent leur taux d'évolution. Cependant Schlötterer et al. (1994) ont montré chez la Drosophile que seule une fraction des ITS possédait une telle fonction : dans ce cas le taux d'évolution des ITS serait proche d'un taux neutre.

Les ITS ont récemment été employés, en combinaison avec des marqueurs mitochondriaux, dans le cas du genre *Reticulitermes* (Jenkins et al., 2001).

Comme l'a souligné Avise en 1994, les marqueurs moléculaires sont souvent utilisés pour confirmer des études phylogénétiques basées sur d'autres caractères telle que la morphologie. Si cette approche peut s'avérer intéressante pour trancher entre deux hypothèses divergentes, une ré-analyse globale des organismes vivants n'est pas nécessaire.

De la recherche à l'application : le cas de Reticulitermes sp. nov.

La présence d'un nouveau phénotype en Isère dans la commune de Domène s'accompagnait de nouvelles mesures à prendre en considération, telles l'adaptation potentielle des traitements face à ce nouveau phénotype ou encore la prospection de nouveaux sites d'infestation. Ce phénotype a d'ailleurs été retrouvé la même année dans une ville du Nord de l'Italie (Bagnacavallo, Bologna).

La présence des termites dans la commune de Domène était connue depuis une trentaine d'années, particulièrement par les habitants du centre ancien. Ce n'est que depuis 1995 que le problème a été réellement pris en compte par la nouvelle municipalité dans un projet global d'amélioration du centre ville.

L'ampleur de l'attaque et la présence d'un nouveau phénotype jusqu'alors jamais rencontré en France ont amené la municipalité de Domène à signer un contrat avec le Laboratoire de Neurobiologie du CNRS. La convention de ce contrat, signée le 22 avril 1999, avant la nouvelle législation sur les termites, prévoyait une étude sur trois ans. Un avenant à cette convention a été signé avec la Mairie de Domène à la suite du déménagement de l'équipe à Tours dans une unité mixte de recherche CNRS-Université (UMR 6035). Le contrat sera terminé fin octobre 2002.

L'étude s'intitule :

"Étude des termites présents à Domène et dans les environs, recherche des moyens de lutte"

Les objectifs de cette étude sont les suivants :

- Étude biologique de l'espèce :

- Origine et position systématique de la nouvelle espèce : détermination de son lieu de provenance grâce à la comparaison (morphologique, comportementale, chimique et génétique) avec d'autres espèces de termites
- Comportement (ouverture / fermeture des colonies) : la capacité d'accepter ou non un individu d'une colonie différente influence directement les résultats d'un traitement **[*Annexe confidentielle p. 27*]**
- Estimation de la densité des populations : une aire d'étude sera employée pour estimer l'aire de fourragement et la densité des colonies de termites par la technique de capture-marquage-recapture **[*Annexe confidentielle pp. 28-30*]**

- Cartographie : mesure de l'étendue de l'infestation largement influencée par les modalités de propagation (essaimage, propagation souterraine, bouturage, transport de matériaux infestés) **[*Annexe confidentielle pp. 30-34*]**

- Traitement : la délimitation des aires contaminées sera suivie par une politique de traitement à vaste échelle. Un cahier de charges sera réalisé pour la mise en place du traitement **[*Annexe confidentielle pp. 34-37*]**
  - Efficacité des toxines existantes sur le marché : les produits anti-termites présents sur le marché seront testés sur la nouvelle espèce par le biais de :
    - tests de laboratoire
    - chantiers expérimentaux sur le terrain.
  - Liaison avec les applicateurs : un aspect important avant le début des traitements est le contact avec les acteurs de la lutte contre les termites. Un suivi sera réalisé avant et après le début de la phase opérationnelle.

Matériel et méthodes

Les techniques employées pour la récolte et l'analyse des données ont été décrites dans les sections "Matériel et méthodes" des différents articles. Dans ce chapitre nous avons intégré les informations fournies et rajouté des concepts d'ordre général où cela se révélait nécessaire.

Matériel biologique

Notre intérêt porte sur la recherche de l'origine et des modalités de propagation d'un nouveau phénotype de termite *Reticulitermes*, ***R. sp. nov.***, identifié en 1998 dans une commune du département de l'Isère. En absence de données sur la distribution de ce phénotype à l'état naturel, j'ai été amené à rechercher des échantillons par étapes en fonction des résultats obtenus au cours de l'étude.

Dans un premier temps, nous avons comparé ce nouveau phénotype aux espèces déjà connues en Europe : ***Reticulitermes grassei***, ***R. banyulensis***, ***R. lucifugus***, ***R. l. corsicus***, ***R. santonensis*** et ***R. balkanensis***.

Pour les cinq premières espèces citées ci-dessus, les colonies prélevées en milieu naturel ont ensuite été placées en salle d'élevage dans leurs souches d'origine. Les échantillons de plus petite taille ont été maintenus dans des boîtes en plastique (format 36x24x14cm), sur un substrat de Sable de Fontainebleau (SDS) et nourris avec du papier filtre (pour les échantillons de petite taille) ou avec du bois (pour les échantillons de taille moyenne).

Comme des résultats préliminaires nous avaient montré de fortes similarités morphologique et chimique entre *R. sp. nov.* et *R. balkanensis* présente en Grèce, j'ai effectué une mission de récolte en Grèce (octobre 2000) afin de récolter cette dernière espèce (les échantillons dont on disposait étaient en effet en mauvais état de conservation). D'autres échantillons de termites provenant de l'aire est-européenne ont pu être étudiés grâce à la collaboration de Dani Simon de l'Université de Tel-Aviv (Israël) et de James Austin de l'Université d'Ankara (Turquie).

Dans un deuxième temps, en raison du caractère urbain de l'infestation causée par *R. sp. nov.*, nous avons porté notre attention sur d'autres localités urbaines grâce à la participation d'un grand nombre d'applicateurs.

La découverte du même phénotype dans d'autres localités dans le sud de la France m'a amené à effectuer une deuxième mission de récolte en Côte d'Azur (juin

2001). Ce sera à la suite de cette récolte qu'on identifiera le premier site non urbain de *R. sp. nov.* dans le Parc de Sophia Antipolis (06).

Morphologie

La morphologie du postclypeus (vues dorsale et latérale) avait été préalablement utilisée pour identifier les espèces de *Reticulitermes* en Europe (Clément, 1978). Ce même caractère a été pris en compte pour le nouveau phénotype. Les images ont été réalisées par microscopie électronique à balayage (JEOL JSM–6100 ; 10 volts) à l'Université d'Aix-Marseille II.

Hydrocarbures cuticulaires

Procédure d'extraction des composés cuticulaires

Le principe de la chromatographie en phase gazeuse est de séparer les produits d'un mélange selon leurs propriétés physico-chimiques, chaque produit étant caractérisé par un temps de rétention spécifique. Selon le nombre d'ouvriers à disposition par point de récolte, 50 à 100 individus sont immergés pendant 10 minutes dans 2 ml de pentane. Le choix du pentane comme solvant avait été déterminé par la nature apolaire des composés cuticulaires à extraire. L'extrait est ensuite concentré sous flux d'azote et 2 µl sont injectés à l'aide d'une seringue Hamilton© dans un chromatographe en phase gazeuse Delsi-Nermag 200DN. L'appareil est équipé d'un détecteur à ionisation de flamme (FID), d'une colonne de type apolaire Chrompack CPSil5 WCOT (25 m de longueur, 0,25 mm de diamètre, 0,25 µm d'épaisseur de phase) et d'un injecteur en mode split/splitless (15 s). L'hélium est le gaz vecteur sous une pression de 1 bar. Le programme de température utilisé consiste en une montée de température de 30°C/min de 70°C à 150°C suivie d'un isotherme de 5 min, puis d'une montée de 5°C/min de 150°C à 320°C, et enfin d'un isotherme de 10 min à 320°C. L'appareil est couplé à un intégrateur Enica 31 pour le calcul des surfaces des différents pics du chromatogramme, les surfaces étant proportionnelles aux quantités des produits.

La détermination des produits cuticulaires des *Reticulitermes* européens avait été réalisée par Bagnères et al. (1990, 1991) par chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC-MS). Pour cette étude, la détermination a été revue et intégrée en collaboration avec A.-G. Bagnères. La technique de GC-MS

fragmente les molécules sortant de la colonne du chromatographe grâce au bombardement d'un faisceau d'électrons de haute énergie. Le spectre de fragmentation résultant apporte des informations structurales telles la longueur de la chaîne d'atomes de carbones, la présence de groupes fonctionnels et la masse moléculaire de la molécule. L'identification des hydrocarbures présents dans les extraits cuticulaires a été réalisée à l'aide d'un chromatographe en phase gazeuse Hewlett-Packard HP5890 Série II (colonne apolaire Chrompack CPSil 5, splitless 1 min, hélium 2 bars) couplé à un spectromètre de masse quadrupôle MS Engine Hewlett-Packard 5989A (source 240°C, interface 300°C), le tout contrôlé par une station MS Chemsystem HPUX, sous système Unix.

Analyse des données

Les produits cuticulaires peuvent être regroupés par classes d'hydrocarbures, nous distinguerons donc les *n*-alcanes, les methyl-alcanes (mono- et diméthyl-alcanes) et les hydrocarbures insaturés (monoènes et diènes). Les surfaces obtenues par l'intégrateur pour chacun des pics sont corrigées par un coefficient de correction K ($K = 0{,}042\ n + 0{,}01$, où n = nb total d'atomes de carbone dans la molécule). Ce coefficient corrige les erreurs induites par le détecteur FID dont la sensibilité varie en fonction de la longueur de la chaîne carbonée de la molécule (Bagnères, 1989 ; Bagnères et al., 1990). Les pics pouvant se confondre avec le bruit de fond et posant des problèmes d'intégration n'ont pas été pris en compte dans les analyses.

Chaque individu est caractérisé par les proportions relatives de chacun des composés présents dans l'extrait cuticulaire. Les valeurs sont ensuite rentrées dans une matrice ayant comme variable qualitative les différents individus. Les données sont standardisées et analysées avec les logiciels Statgraphics v. 6 et UniWin v. 3.01 (UniStat II).

Analyse en composante principale (ACP)

L'analyse en composante principale est une méthode descriptive qui donne une représentation synthétique des données en minimisant la perte d'informations, c'est-à-dire en extrayant le maximum de variance à partir des données. Cette technique est basée sur les valeurs de corrélation linéaire entre les variables (=composés cuticulaires).

La corrélation entre deux variables peut être représentée par une droite de régression, la valeur de la corrélation étant proportionnelle à la variance expliquée par la droite. On peut donc définir une troisième variable qui résulte de la combinaison linéaire des deux variables de départ et qui approche la droite de régression. L'extension de cet exemple au cas de variables multiples permet l'extraction d'un nombre inférieur de composantes par rapport aux variables d'origine. Le pourcentage de variance expliquée par chaque composante est donnée par la valeur propre (eigenvalue). Chaque variable (=composé cuticulaires) est caractérisée par un coefficient de corrélation (r) avec chacun des axes. Les variables participent d'autant plus à la formation des axes que le coefficient de corrélation correspondant est proche de 1 ou de –1. Ainsi, les individus projetés sur les parties négatives ou positives des axes sont caractérisés par les variables qui sont le plus fortement corrélées avec ces mêmes parties des axes. Le nombre minimal de composantes à utiliser est donnée par le scree-test: la variance expliquée (valeur propre ou eigenvalue) est représentée en fonction du nombre de composantes. Le nombre de composantes à utiliser est donné par le point qui précède le plateau : au-delà la contribution des facteurs est négligeable.

Analyse génétique moléculaire

Dans le cadre de l'étude des relations phylogénétiques au sein du genre *Reticulitermes* en Europe, nous avons utilisé la comparaison de séquences d'ADN mitochondrial et nucléaire.

Les caractéristiques de chacune des régions d'ADNmt et nucléaire analysées, le protocole d'extraction de l'ADN ainsi que les paramètres d'amplification et de séquençage sont décrits dans les sections "Matériels et Méthodes" des différents articles.

Analyse phylogénétique

Terminologie

Pour éviter toute ambiguïté sur l'utilisation des termes relatifs aux différents types d'arbres phylogénétiques présentés, nous allons préciser la signification des termes employés (Clewley, 1998 ; Kitching et al., 1998) :

cladogramme : indication des relations hiérarchiques entre les taxa, mais absence d'un axe temporel ;

phylogramme : indication des relations hiérarchiques et présence d'un axe temporel.

Alignement des séquences

Les séquences consensus ont été alignées avec l'algorithme Clustal W (Thompson et al., 1994) inclus dans l'éditeur de séquences BioEdit 4.8.10 (Hall, 1999) et corrigées manuellement. Des analyses phylogénétiques préliminaires ont été réalisées avec les logiciels PHYLIP 95 (Felsenstein, 1993) ; successivement les séquences nucléotidiques ont été converties au format NEXUS et analysées avec le logiciel PAUP 4.0b10 (Swofford, 2001) car il permet de contrôler un plus grand nombre de paramètres.

La reconstruction phylogénétique est basée sur la distinction entre caractères apomorphiques (dérivés) et plésiomorphiques (primitifs). Plusieurs méthodes on été proposées pour déterminer l'apomorphie et la plésiomorphie des caractères : elles peuvent être classées en méthodes directes, basées sur les informations disponibles à partir des espèces étudiées (ontogenèse des caractères), et méthodes indirectes, qui demandent une source d'information externe au groupe étudié. La comparaison avec une ou plusieurs espèces *outgroup* constitue la méthode indirecte normalement employée dans les études phylogénétiques (Kitching et al., 1998). L'*outgroup* étant défini comme l'espèce externe la plus proche aux taxa étudiés, nous avons utilisé une espèce de termite souterrain de la sous-famille des Coptotermitinae (Rhinotermitidae), *Coptotermes formosanus*, à partir de laquelle le genre *Reticulitermes* aurait évolué (Krishna, 1970).

Un fragment de 11 bases, correspondant à une élongation chez l'espèce outgroup, n'a pas été inclus dans les analyses car ne possédant pas d'information phylogénétique. Les *gaps* ont été considérés comme valeurs manquantes.

Méthodes de reconstruction phylogénétique

La reconstruction d'un arbre peut être définie comme l'inférence statistique de la phylogénie réelle qui, elle, est inconnue. Ce concept d'inférence inclut à la fois l'estimation de la topologie de l'arbre et de la longueur des branches. Le problème majeur consiste dans l'estimation de la topologie, la longueur des branches étant relativement simple à estimer une fois la topologie déterminée (Nei, 1996).

Nous avons analysé les séquences avec trois différentes méthodes dans le but de pouvoir comparer les résultats obtenus : méthode du *Neighbor-Joining* (NJ), *Maximum de Parcimonie* (MP) et *Maximum de Vraisemblance* (Maximum Likelihood, ML).

Ces méthodes ont été décrites en détail par Nei (1987, 1996), Felsenstein (1988), Avise (1994) et Swofford et Olsen (1990), et seront expliquées brièvement dans les paragraphes suivants.

Neighbor-Joining (NJ)

Il s'agit d'une méthode basée sur la création d'une matrice de distances entre les espèces (Saitou et Nei, 1987). C'est une version simplifiée de la méthode de *Minimum Evolution* (ME) où la longueur des branches est estimée pour chacune des topologies réalisables. Cette méthode a donc l'avantage de réduire considérablement le temps de calcul, tout en gardant une probabilité importante d'obtenir la topologie réalisée avec la méthode ME pour des séquences supérieures à 500 bases (Nei, 1996). La méthode de NJ est préférable aux autres car elle ne suppose pas l'existence d'une horloge moléculaire (Saitou, 1996) : les branches peuvent avoir différentes longueurs ce que n'est pas permis avec d'autres algorithmes (cas de l'UPGMA *Unweighted Pair-Group method with Arithmetic Mean*).

Les inconvénients des méthodes de distance consistent dans la perte d'information associée à la transformation d'un alignement de séquences en matrice de distance et l'impossibilité de traiter des modèles dont les paramètres ne sont pas connus *a priori* (Whelan et al., 2001).

Maximum de Parcimonie (MP)

Le principe de cette méthode est de réduire le nombre de substitutions nécessaires pour obtenir la topologie finale, en d'autres termes on préfère l'explication la plus parcimonieuse. L'absence d'hypothèses sur le modèle d'évolution et la possibilité d'incorporer plusieurs types d'informations (position, taux de transition et transversion...) ont rendu cette méthode très populaire dans les années 1970. Actuellement on préfère les méthodes de maximum de vraisemblance (voir paragraphe suivant) car plus consistantes[1] (Steel et Penny, 2000). La probabilité

[1] La consistance statistique est définie comme la probabilité d'obtenir la topologie correcte avec un nombre infini de données.

d'obtenir le cladogramme réel est inversement proportionnelle à la divergence entre les séquences (augmentation de l'homoplasie). La présence de branches de longueurs très différentes peut également amener à une topologie finale incorrecte (Stewart, 1993).

Maximum de Vraisemblance (Maximum Likelihood, ML)

Cette méthode est basée sur un modèle d'évolution et sur la capacité du phylogramme obtenu de décrire les données de départ. Pratiquement la méthode de ML sélectionne le phylogramme qui maximalise la probabilité d'obtenir les données observées sur la base du modèle choisi.

Il s'agit d'une méthode lourde d'un point de vue des calculs. Son intérêt réside dans son support statistique qui permet la réalisation de tests d'hypothèse et dans sa consistance statistique.

Le choix du modèle a été réalisé avec le logiciel MODELTEST (Posada et Crandall, 1998) qui réalise une routine de *likelihood ratio tests* (Huelsenbeck et Crandall, 1997 ; Huelsenbeck et Rannala, 1997) entre le modèle plus simple et celui plus complexe. Si l'ajout de paramètres augmente la probabilité (=likelihood), le modèle plus complexe est sélectionné.

Ce logiciel permet également de tester pour chaque modèle l'effet de la correction gamma (Yang, 1993, 1996) et de l'estimation de la proportion de sites invariables.

Évaluation du support interne

Une approche intuitive pour évaluer le support d'un groupe monophylétique consiste dans le comptage des synapomorphies (caractères dérivés communs) pour le groupe donné. En d'autres termes, la longueur d'une branche pourrait être considérée comme proportionnelle au support de la branche (groupe monophylétique). Mais cette approche ne considère pas, par exemple, la présence de substitutions inverses qui rendent plus difficile la détection des synapomorphies. Dans ce contexte, on utilise le concept d'homoplasie pour définir une similarité qui ne provient pas de l'héritage d'un ancêtre commun, mais qui résulte d'un phénomène de convergence, parallélisme, analogie ou réversion. En présence d'homoplasie, la longueur des branches ne peut pas être employée comme une estimation du support.

Parmi les méthodes proposées pour estimer le support d'un groupe (recensées par Felsenstein, 1988) nous avons utilisé le *bootstrap non paramétrique*. Il s'agit d'un test statistique basé sur un échantillonnage avec remise des caractères pour réaliser un "data set" avec les mêmes dimensions de l'original (Felsenstein, 1985).

Le *bootstrap* a été initialement introduit comme une mesure de la répétabilité d'une analyse phylogénétique (la probabilité d'obtenir un groupe spécifique sur la base d'un échantillon indépendant de caractères). Cependant il est normalement employé comme une mesure de la précision (*accuracy*), c'est-à-dire la probabilité d'obtenir la branche réelle. Hillis et Bull (1993) ont étudié la relation entre ces deux facteurs sur la base de simulations. Ils observent que toutes les branches internes avec des valeurs de bootstrap supérieures à 80% définissent un vrai clade, et plus de 95% des clades avec une valeur de bootstrap supérieures à 70% sont corrects.

Cette valeur de 70% est ainsi devenue la valeur d'usage courant pour identifier une monophylie bien supportée. Elle sera donc employée comme seuil discriminant dans notre étude.

Pour les analyses de distances et de maximum de parcimonie, nous avons réalisé 2000 réplications, permettant d'estimer la valeur de bootstrap obtenue après un nombre infini de réplications avec une probabilité de 95% (Hedges, 1992). Pour les analyses avec le maximum de vraisemblance, seules 100 réplications ont été générées pour réduire les temps de calcul.

Le *bootstrap* a été réalisé avec un réarrangement des branches (option TBR et MULTREES dans le logiciel PAUP) car il permet d'obtenir des estimations plus précises des valeurs de *bootstrap* par rapport au *bootstrap simple* (sans réarrangement des branches) et par rapport à d'autres méthodes d'échantillonnage comme le *jackknife* (Debry et Olmstead, 2000 ; Mort et al., 2000).

Utilisation de contraintes

Pour tester la position systématique de *R. sp. nov.* nous avons défini des contraintes, notamment pour valider une éventuelle relation *R. speratus* - *R. sp. nov.* (Marini et Mantovani, 2002). Ces contraintes ont été appliquées aux analyses de Maximum de Parcimonie avec le test de Templeton (1983) et de Maximum de Vraisemblance avec le *bootstrap paramétrique* (Hillis et al., 1996). La procédure a été décrite en détail dans **l'article I**.

Visualisation des arbres phylogénétiques

Les arbres phylogénétiques obtenus avec PAUP ont été visualisés avec le logiciel TreeView (v.1.6.1.) (Page, 1996). Les arbres ont été enracinés avec l'espèce *outgroup Coptotermes formosanus*. Les indices de consistance (CI ; Kluge and Farris, 1969) et de rétention (RI ; Farris, 1969) ont été calculés pour les cladogrammes obtenus par Maximum de Parcimonie. Le CI est une mesure directe de l'homoplasie tandis que le RI mesure la similarité dans les caractères en terme de synapomorphie. Les deux indices varient entre 0 (homoplasie totale) et 1 (absence d'homoplasie).

Analyse "Total Evidence"

Une première étude basée sur l'analyse séparée des hydrocarbures cuticulaires et de séquences d'ADN mitochondrial a été présentée par Jenkins et al. (2000) pour établir les relations phylogénétiques entre des espèces sympatriques de *Reticulitermes* présentes aux États-Unis. Clément et al. (2001) ont comparé les *Reticulitermes* européens avec une approche multidisciplinaire (morphologie, hydrocarbures cuticulaires, ADNmt), mais jusqu'à présent aucun travail n'avait été réalisé avec une analyse combinée (*Total Evidence*) hydrocarbures cuticulaires-séquences d'ADNmt.

Le débat analyse combinée vs. analyse séparée a commencé à la suite de la publication de Kluge (1989) où l'auteur avait suggéré d'employer de façon générale la totalité des caractères dont on disposait pour optimiser leur caractère informatif : les données (gènes, morphologie), soumises à des contraintes différentes, ont des taux d'évolution différents, et peuvent interagir pour augmenter le support de la topologie de l'arbre à différents niveaux.

Une autre approche a été proposée par Miyamoto and Fitch (1995) qui suggèrent d'analyser les partitions de données séparément afin d'obtenir plusieurs estimations indépendantes des arbres : la congruence des topologies fournit un support à la structure phylogénétique obtenue (congruence taxonomique). Un compromis entre "analyse combinée" et "congruence taxonomique" a été proposé par Bull et al. (1993) (*conditional data combination*). Les partitions sont tout d'abord analysées séparément, et ensuite soumises à un test d'homogénéité : si le test n'est pas significatif, alors les partitions produisent des arbres phylogénétiques qui ne sont pas en conflit entre elles, et peuvent être combinées et analysées simultanément. Parmi les méthodes

proposées pour tester l'homogénéité des partitions (Huelsenbeck et al., 1996) nous avons employé le test proposé par Farris et al. (1995).

Résultats et Discussion

Systématique moléculaire de Reticulitermes sp. nov.

Suite à l'identification d'un nouveau phénotype de *Reticulitermes* dans l'Isère, le nombre d'espèces de termites en France était remis en question :

S'agissait-il d'une nouvelle espèce ?

Les résultats présentés ici concernent plus particulièrement la position systématique de ce nouveau phénotype au sein du genre *Reticulitermes*.

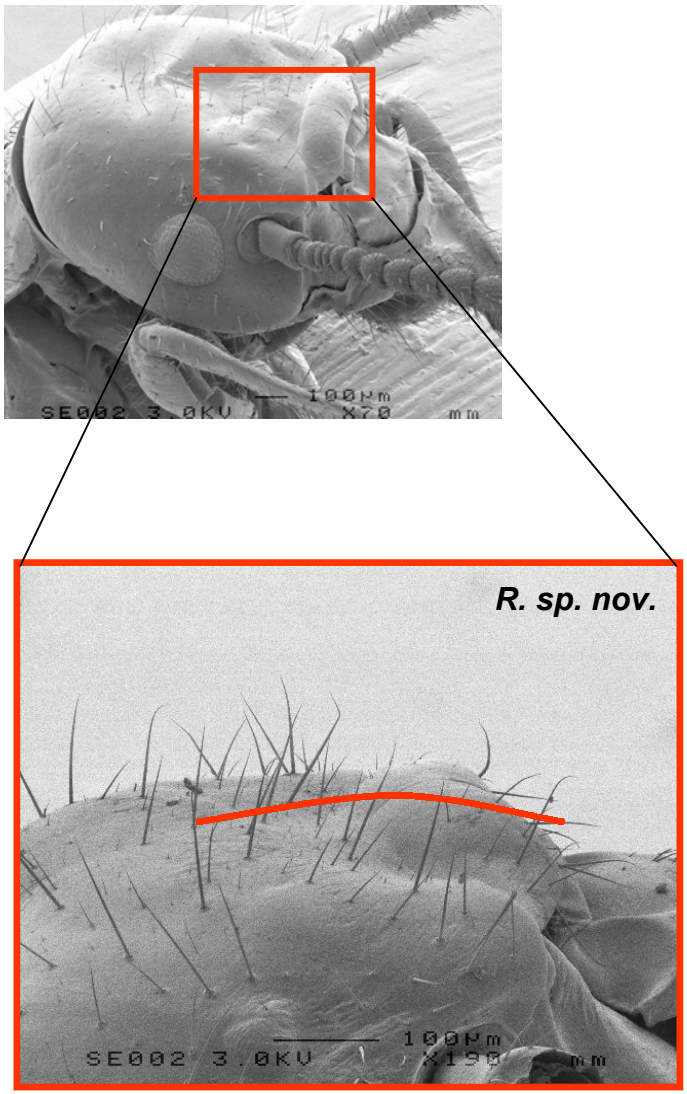
Dans un premier temps, nous avons examiné la morphologie du post-clypeus par microscopie électronique à balayage, et nous l'avons comparée aux autres espèces présentes en Europe (figure 6). Il s'agit d'un caractère dont la validité diagnostique au niveau spécifique avait déjà été confirmée par Clément (1978) et Bagnères (1989).

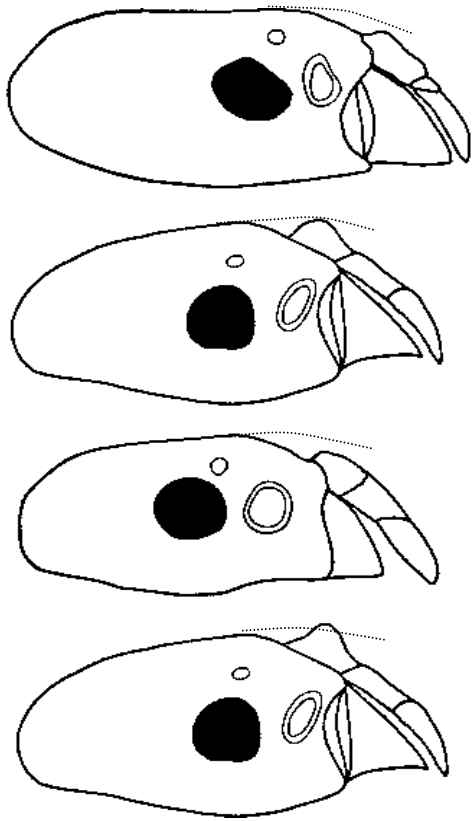
La similarité morphologique avec *R. balkanensis* et la découverte de nouveaux sites présentant ce nouveau phénotype dans le Sud de la France m'a conduit à élargir la prospection en France méridionale et dans l'est de l'Europe. Dans cette dernière région, l'échantillonnage était d'autant plus nécessaire qu'une certaine confusion taxonomique y régnait puisque les études les plus récentes employaient encore l'ancienne classification de Rossi (1792).

Dans **l'article I**, nous avons utilisé des séquences d'ADN mitochondrial et nucléaire (ND1-16S et ITS2 respectivement) pour établir les relations phylogénétiques au sein du genre *Reticulitermes* et étudier l'évolution du nouveau phénotype sur la base de certaines hypothèses phylogénétiques. La monophylie de certains groupes a également été testée par le biais d'un *bootstrap* paramétrique.

Les résultats obtenus confirment une origine de *Reticulitermes sp. nov.* à partir des populations est-européennes suite à une migration via des routes de colonisation post-glaciaires (figure 7). Son identité spécifique a également été confirmée.

L'**article II** est le résultat d'une collaboration avec James Austin de l'Université d'Ankara (Turquie). Dans cette étude, les variations génétiques entre les différents *Reticulitermes* ont été étudiées grâce à la comparaison de séquences mitochondriales





R. santonensis

R. banyulensis

R. grassei + R. lucifugus

R. balkanensis + R. sp. nov.

Clément et al., 2001

Figure 6. Morphologie du post-clypeus chez *Reticulitermes sp. nov.* et comparaison avec les autres *Reticulitermes* européens (Clément et al., 2001).

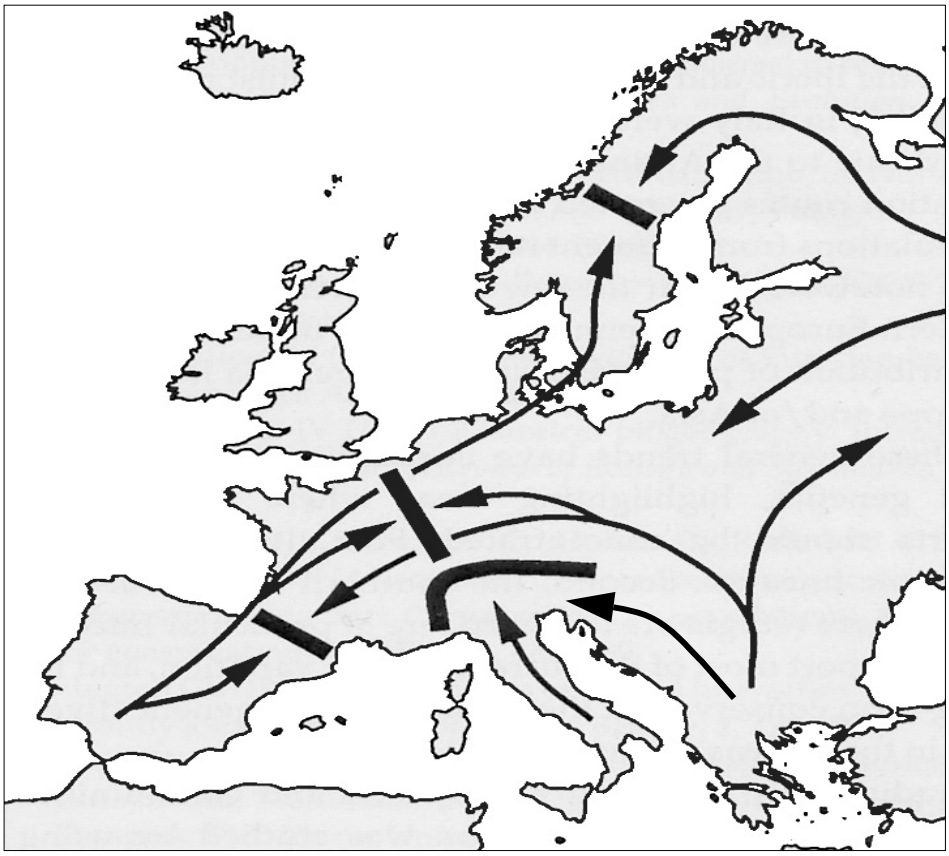


Figure 7. Routes principales de colonisation postglaciaires (d'après Taberlet, 2001)

du gène COII. Les données montrent une monophylie et une forte similarité pour les échantillons provenant de la "région" Balkans-Turquie-Israël. Ce groupe apparaît bien séparé des autres espèces européennes. La position du nouveau phénotype *R. sp. nov.* est en adéquation avec les résultats obtenus à partir des autres marqueurs moléculaires.

L'avancement des connaissances moléculaires peut ainsi contribuer à établir un tableau exhaustif de la systématique des *Reticulitermes* européens. La disponibilité de nouvelles données nous montre également qu'il est nécessaire de réviser la systématique dans l'Est de l'Europe qui, à l'état actuel, ne reflète pas l'histoire évolutive des espèces.

- **Article I** : **Uva, P.**, J.-L. Clément, J.W. Austin, J. Aubert, V. Zaffagnini, A. Quintana and A.-G. Bagnères. The origin of a new *Reticulitermes* termite (Isoptera, Rhinotermitidae) inferred from mitochondrial and nuclear DNA data. Soumis à *Molecular Phylogenetics and Evolution*.
- **Article II** : J.W. Austin, A.L. Szalanski, **P. Uva**, A.-G. Bagnères and A. Kence. A comparative genetic analysis of the subterranean termite genus *Reticulitermes* (Isoptera: Rhinotermitidae). Accepté pour publication in *Annals of the Entomological Society of America.*

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Origin of a new *Reticulitermes* termite (Isoptera, Rhinotermitidae) inferred from mitochondrial and nuclear DNA data

Paolo Uva,[a] Jean-Luc Clément,[a] James W. Austin,[b] Josiane Aubert,[c] Valeria Zaffagnini,[d] Alexandre Quintana,[e] and Anne-Geneviève Bagnères[a,*]

[a] *Institut de Recherche sur la Biologie de l'Insecte, UMR CNRS 6035, Université F. Rabelais, Parc Grandmont, 37200 Tours, France*
[b] *Department of Entomology, University of Arkansas, Fayetteville, 72701 AR, USA*
[c] *Département Génie Biologique, Université de Toulon et du Var, BP132, 83957 La Garde, France*
[d] *DowAgrosciences, 40123 Bologne, Italy*
[e] *Institut de Recherche Pierre-Fabre, Centre de Recherche sur les Substances Naturelles, UMR CNRS 1973, Parc Technologique du Canal, 31527 Ramonville, France*

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Abstract

The Holoarctic termite genus *Reticulitermes* is widely distributed in Europe. A new *Reticulitermes* species, *R. sp. nov*, was recently found in France and Italy. Its phylogenetic position was investigated using a 743-bp fragment of mitochondrial 16S rRNA-ND1 genes and 382-bp of the nuclear ITS2 region. Phylogenies for these sequences were estimated by neighbor-joining, maximum-parsimony and maximum-likelihood analysis. The results strongly supported a relationship between *R. sp. nov*. and the termite species from the eastern Mediterranean area including *Reticulitermes balkanensis* from the Balkans, *Reticulitermes lucifugus* from Turkey and *Reticulitermes clypeatus* from Israel. The hypothesis of a relationship between *R. sp. nov*. and the Japanese *Reticulitermes speratus* was rejected by parametric bootstrap. The current distribution of *R. sp. nov*. could be linked to postglacial colonization routes between Balkan refuge and northern regions.

Keywords: Mitochondrial DNA; ND1; ITS2; Termites; *Reticulitermes*; Parametric bootstrap

1. Introduction

The nearly 2000 species of Isoptera in the world (Kambhampati and Eggleton, 2000) play an important natural role in the breakdown of cellulose in natural environments. However they are highly destructive pests causing extensive damage in urban settings. The cost of treatment against termites in Europe is expected to top 1 billion euros by 2005 (UNEP and FAO, 2000).

Reticulitermes is a Holoarctic genus whose distribution in the Palearctic region is the result of historical climatic events, i.e., glaciation during the Quaternary period (Clément, 1978), as well as current factors, i.e., environmental conditions and human activity. They are subterranean termites, whereby their colonies usually consist of galleries constructed below ground level. Although *Reticulitermes* can feed on living tissue, their chief food source is dead wood and other cellulose-containing items that workers collect and carry back to the colony. This reclusive lifestyle has hindered studies of colonial social structure. New colonies are founded either by a pair of swarming winged reproductives and/or by secondary reproductives cut off from the main colony.

Rossi (1792) described the first *Reticulitermes* termites in the western Palearctic region from samples collected in Italy. Subsequently other species were identified based on morphology, behavior, cuticular hydrocarbons, enzymatic polymorphism, and mitochondrial DNA (mtDNA) (Clément, 1978; Clément et al., 2001), including *Reticulitermes grassei* and *Reticulitermes banyulensis*

* Corresponding author. Fax: +33-2-47-36-73-56.
E-mail address: bagneres@univ-tours.fr (A.-G. Bagnères).

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in France and Spain, *Reticulitermes balkanensis* in the Balkans, *Reticulitermes lucifugus* in Italy and France, and the *Reticulitermes lucifugus corsicus* subspecies on the islands of Corsica (France) and Sardinia (Italy). Recent studies have provided evidence of a transtyrrenian distribution of the Corsican subspecies due either to paleogeographical events or human intervention (Marini and Mantovani, 2002; Uva et al., unpublished data). According to some authors, *Reticulitermes santonensis* found in France and some urban areas of Europe is synonymous with North American *Reticulitermes flavipes* (Bagnères et al., 1990; Clément et al., 2001; Jenkins et al., 2001; Marini and Mantovani, 2002; Vieau, 2001; Dronnet et al., unpublished data).

The taxonomy of termites in the eastern Mediterranean area remains obscure. In his review Harris (1970) stated that all *Reticulitermes* found in this area were *R. lucifugus* as classically defined by Rossi (1792) except for those found in Israel which were *Reticulitermes clypeatus* Lash (1952). Thus the name *R. lucifugus* is currently applied to two distinct species from Italy and Turkey. To avoid any confusion due to this homonymy, we will use the name *R. lucifugus* for populations in Italy where the species was first described (Rossi, 1792) and the name *R. l.*-Turkey for population in Turkey. A recent study should clarify the exact classification of *Reticulitermes* termites in these regions (Austin et al., 2002).

In a recent paper on termite species description rates, Eggleton (1999) reported that the cumulative number of species description in the western Palearctic region had leveled off considerably since 1949. This finding suggests that most of the termite species have been identified in this area where terrestrial fauna has been exhaustively studied. On the assumption that the classification of termites was definitive, samples have been identified based on geographic origin alone. Some *Reticulitermes* samples showing phenotypical differences from cogeneric European species have been classified as *R. lucifugus* simply because they were collected in Italy (Springhetti, 1965; Marini and Ferrari, 1998). However since further study has revealed significant differences with other *Reticulitermes* species with regard to morphology, behavior and cuticular hydrocarbons, these samples have been reclassified as a separate entity designated as *R. sp. nov*. (Bagnères, pers. commun.; Clément et al., 2001). Afterwards, we identified termites displaying the *R. sp. nov*. phenotype in urban areas located in northern and southern Italy, in southeastern France and in one natural site located on the French Riviera (Uva et al., 2002).

The exact relationship of *R. sp. nov*. with other *Reticulitermes* species is uncertain. Based on mtDNA sequences, Marini and Mantovani (2002) proposed kinship with the Japanese *R. speratus*. However Clément et al. (2001) found similarities between *R. sp. nov*. and *R. balkanensis*, *R. santonensis* (cuticular hydrocarbons) and *R. clypeatus* (morphology).

Previously mtDNA was used to study phylogenetic relationships within the *Reticulitermes* genus (Jenkins et al., 2001; Marini and Mantovani, 2002) and to reveal matriarchal genetic structure of *Reticulitermes* colonies in North America (Jenkins et al., 1998). Moreover, a recent study for the *Reticulitermes* genus that included the internal transcribed spacer sequence of a nuclear ribosomal RNA (Jenkins et al., 2001) provided an indication of the utility of this nuclear marker for closely related species, as previously pointed out by other authors (Schlötterer et al., 1994; Vogler and DeSalle, 1994). In this study we attempted to use both mtDNA and ribosomal ITS2 to clarify the ambiguous morphological and chemical findings concerning the phylogenetic relationship between *R. sp. nov*. and western Palearctic *Reticulitermes* termites, to assess its taxonomic status and to provide information in the general pattern of *R. sp. nov*. evolution. We also tested several hypotheses of monophyly for the termites in the eastern Mediterranean area.

2. Materials and methods

2.1. Samples

Various *Reticulitermes* samples from different Mediterranean countries (Fig. 1) were studied. DNA extraction was performed either immediately after collection in the field or after storage in alcohol at −20 °C. In addition to Mediterranean *Reticulitermes*, *R. speratus* from Japan (Ibaraky) and *R. flavipes* from North America (Raleigh, NC) were included in the study. Full information concerning the samples is given in Table 1. To allow for intraspecific variations, two or three specimens were included for each species. A total of 42 specimens belonging to 20 taxa were analyzed. Sequences of *R. lucifugus* were drawn from GenBank for comparison (Uva et al., unpublished data).

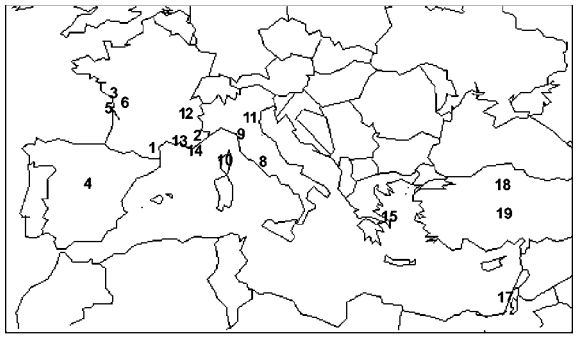

Fig. 1. Map of *Reticulitermes* collection sites in Europe. See Table 1 for reference numbers.

Table 1
Sampling Information and GenBank Accession Numbers

No.	Species name	Collection site	Sample code	GenBank Accession No.	
				ITS2	ND1
1	*Reticulitermes banyulensis*	Beziers (34-France)	Rb-Bez	AY140912	AY101825
2	*R. banyulensis*	Vidauban (83-France)	Rb-Vid	AY140913	AY101826
3	*R. grassei*	Forêt de la Coubre (17-France)	Rg-For	AY140914	AY101827
4	*R. grassei*	Aranda de Duero (Spain)	Rg-Ad	AY140915	AY101828
5	*R. santonensis*	Ile d'Oleron (17-France)	Rs-Ole	AY140916	AY101829
6	*R. santonensis*	Tonnay-Charente (17-France)	Rs-Tc	AY140917	AY101830
7	*R. flavipes*	Raleigh (USA)	Rf	AY140918	AY101831
8	*R. lucifugus*	Campo di Mare (Italy)	Rl-Cdm	AY140930	AF458623
9	*R. lucifugus*	Viareggio (Italy)	Rl-Via	AY140931	AF458615
10	*R. l. corsicus*	Corsica (France)	Rlc-Cor	AY140919	AY101832
11	*R. sp. nov.*	Bagnacavallo (Italy)	Rsp-Bag	AY140920	AY101833
12	*R. sp. nov.*	Domène (38-France)	Rsp-Dom	AY140921	AY101834
13	*R. sp. nov.*	Château Gombert (13-France)	Rsp-Gom	AY140922	AY101835
14	*R. sp. nov.*	Sophia Antipolis (06-France)	Rsp-Sa	AY140923	AY101836
15	*R. balkanensis*	Dionissos (Greece)	Rbk-Dio	AY140924	AY101837
16	*R. speratus*	Ibaraki (Japan)	Rspe	AY140925	AY101838
17	*R. clypeatus*	Ben Shemen (Israel)	Rcly	AY140926	AY101839
18	*R. l.*-Turkey	Konya (Turkey)	Rl-Kon	AY140927	AY101840
19	*R. l.*-Turkey	Ankara (Turkey)	Rl-Ank	AY140928	AY101841
20	*Coptotermes formosanus*	Baton Rouge (USA)	Cf	AY140929	AY101842

Note. Numbers in the first column refer to the map in Fig. 1.

2.2. DNA extraction, amplification, and purification

Total DNA was extracted from a single termite head using a modified version of the method described by Kocher et al. (1989).

PCR amplification was performed with a Biometra 96 T1 thermal cycler in a 50 μl reaction containing 37.75 μl of distilled H_2O, 5 μl of 10× *Taq* DNA polymerase buffer (500 mM KCl, 100 mM Tris–HCl, and 15 mM $MgCl_2$, pH 9.0), 1 μl of dNTPs (10 mM each dNTP), 2.5 μl of 10 μM solution of each primer, 1.25 U of *Taq* polymerase (Qbiogene), and 1 μl of DNA template. The primers used are shown in Table 2. The amplification profiles were: ND1—2 min denaturation at 92 °C followed by 40 cycles of 15 s at 92 °C, 45 s at 50 °C, 2 min at 62 °C, and a 7 min final extension at 62 °C; ITS2—2 min denaturation at 92 °C followed by 30 cycles of 30 s at 92 °C, 45 s at 50 °C, 45 s at 72 °C, and a 7 min final extension at 72 °C. Both strands of each sample were sequenced. Nucleotide sequences were entered in the GenBank database (see Table 1 for the accession numbers).

2.3. Phylogenetic analysis

Consensus sequences were aligned using the Clustal W algorithm (Thompson et al., 1994) from the BioEdit 4.8.10 sequence editor (Hall, 1999), and corrected manually. Sequence data were analyzed using the PAUP 4.0b10 package (Swofford, 2001).

To polarize the phylogenetic trees, i.e., distinguish plesiomorphic from apomorphic states, we used one subterranean species of the Coptotermitinae subfamily (Rhinotermitidae), *Coptotermes formosanus* Shiraki, from which the *Reticulitermes* genus reportedly evolved (Krishna, 1970). Trees were drawn using TreeView (Page, 1996).

The DNA sequences were analyzed by neighbor-joining (NJ) method (Saitou and Nei, 1987). Genetic distances were corrected according to the transition/transversion rate (Kimura's two-parameter method, Kimura, 1980). Bootstrap confidence values were calculated from 2000 replications.

Maximum parsimony (MP) analyses were performed using a heuristic search with 100 random-addition

Table 2
Primers used for PCR amplification and sequencing

Name	Sequence (5′–3′)	Source
ND1-F	CTG TTC GAT CAT TAA AAT CTT AC	Aubert, unpublished data
ND1-R	ATC AAA AGG AGC TCG ATT AGT TTC	Aubert et al. (1999)
ITS2-F	TGT GAA CTG CAG GAC ACA T	Jenkins et al. (2001)
ITS2-R	GAC TAC CCC CTA AAT TTA AGC	Jenkins et al. (2001)

replications and Tree-Bisection-Reconnection (TBR) as the branch-swapping algorithm under the assumption of equal weight for all changes. Starting trees for branch swapping were obtained by stepwise addition. Branches were collapsed if maximum branch length was zero. Gaps were treated as missing data. Clade stability was estimated by nonparametric bootstrapping (Felsenstein, 1985), with 2000 replications as suggested by Hedges (1992) for reliable estimation of bootstrap support. Branches having bootstrap node support less than 50% were collapsed.

Prior to maximum likelihood (ML) analyses, the MODELTEST program (Posada and Crandall, 1998) was used to choose the DNA substitution model best fitting our data. Using a series of likelihood ratio tests (Huelsenbeck and Crandall, 1997), the program implements a model test routine between the simpler (null hypothesis) and the more parameter-rich model (alternative hypothesis). If the alternative model significantly improved the likelihood score, the parameters were added to the model. After selection, addition of the gamma correction for different rates over sites (Yang, 1993, 1996), and estimation of proportion of invariable sites were tested to improve the model. Heuristic tree searches were performed with TBR branch swapping with starting trees obtained by stepwise addition. Branches of zero length were collapsed. Nonparametric bootstrap analysis with 100 replicates was performed.

We used two different data sets for phylogenetic analyses: the nuclear ITS2 region and the mitochondrial ND1-16S region. Before being combined, ND1 and 16S data partitions were checked using the partition homogeneity test (Farris et al., 1995) implemented in PAUP (1000 replicates). The tRNA sequences were not included in the alignment.

Some additional analyses were performed for the ND1-16S data set by defining constraints to compare alternative phylogenetic hypotheses. Constraints were enforced in PAUP searches for minimum length trees under the hypothesis of *Reticulitermes speratus* basal to *R. sp. nov*. (accidental introduction from *R. speratus*, as proposed by Marini and Mantovani, 2002) and to the *R. sp. nov., R. clypeatus, R. l.*-Turkey, and *R. balkanensis* group (origin from a common ancestor in the eastern Palearctic area).

Optimal (H_1) and constrained (H_0) ML trees were compared by parametric bootstrapping that produced a null distribution of likelihood ratios between the two hypotheses obtained based on replicate data sets. This was accomplished using the following procedure (Hillis et al., 1996). First ML searches (constrained and unconstrained tree) were conducted in PAUP using the model previously chosen by MODELTEST. After the difference in log likelihood score between trees (observed δ) were recorded, 100 replicates were generated with SeqGen 1.2.5 (Rambaut and Grassly, 1997) based on the constrained ML tree and with the previous model of evolution. Finally the replicate data sets were analyzed with PAUP (constrained and unconstrained) and differences plotted in a histogram. PAUP instruction blocks are available upon request.

For the ND1-16S data partition, genetic pairwise distances were evaluated using the distance matrix option in PAUP according to the method described by Tamura and Nei (1993).

The molecular clock behavior of the ND1 gene in this data set was tested by comparing likelihood scores obtained for trees with and without enforcement of the molecular clock hypothesis. The molecular clock hypothesis was retained if likelihood scores were not significantly different and topologies were equivalent (Huelsenbeck and Rannala, 1997).

3. Results

3.1. Phylogenetic analysis

3.1.1. 16S rRNA-ND1 partial sequences

PCR amplification yielded a fragment of 743 bp (12.147–12.892 in *Drosophila yakuba*, Clary and Wolstenholme, 1985) containing a part of the ND1 gene, tRNA Leu, and a part of the 16S rRNA gene. The tRNA sequences and a highly variable block corresponding to an extension in the outgroup species were excluded from the alignments. The partition homogeneity test (ND1 and 16S) showed a p value of 0.82. Based on this finding, the null hypothesis cannot be rejected (homogeneous partitions) and data were combined for phylogenetic analysis. Moreover, no intraspecific differences in sequences were observed. In the combined data set (667 bp), among the 19 ingroup sequences obtained, there were 134 (20.1%) variable sites and 17 haplotypes were detected (Table 3).

The MP heuristic search produced four trees of equal length (276 steps, CI = 0.701, and RI = 0.722). The strict consensus tree (Fig. 2a) showed some collapsed nodes, but terminal branching patterns were well supported (bootstrap values $\geqslant 70$). Four groups were clearly distinguishable. Group 1 included *Reticulitermes sp. nov.* from Italy and France clustering with *R. balkanensis* (Balkans), *R. l.*-Turkey, *R. clypeatus* (Israel) and *R. speratus* (Japan). Group 2 was made up of *R. banyulensis* from France and *R. grassei* from Spain and France. Group 3 comprised *R. lucifugus* from Italy and the Corsican sub-species *R. l. corsicus*. Group 4 contained *R. santonensis* from France clustering with the North-American *R. flavipes*. Observed polytomy derived from variations in group 3 branch topology which was basal to the group 1 in some trees and clustered with group 2 in others.

MODELTEST selected the Tamura–Nei model (1993) with among-site rate heterogeneity (TrN + G) as

Table 3
Variable sites in partial 16S rRNA-ND1 and complete ITS2 sequences

Colony	16S rRNA	ND1
	1 1 2 6 6 6 6 7 9 1 1 2 2	2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3
	1 7 8 3 1 4 5 8 3 3 2 5 1 3	0 9 5 1 3 9 0 1 2 3 5 7 8 5 6 8 9 3 7 9 3 4 7 2 5 8 7 0 3 9 1 8 1 5 6 1 2 3 6 8 2 5 8 0 4 7 8 1 3 6 7 2 5 8 0 1 3 5 7
Rb-Bez	T C T T A G T T G G T A A	G G T A T G T T G G T C T T T T T G T C A G T G C C T G G G G T G T A T T C G C T T G A A T G T T G T G T C A T G T
Rb-Vid	. . . . .	. . . . . G . . . . .
Rg-For	. . . . . A . A . G . .	. . . . . A . . . . . A T . . . . . A . . . . . G . . . . A .
Rg-Ad	. . . . . A . A . G . .	. . . . . A . . . . . A T . . . . . A . . . . . G . . . . A .
Rs-Ole	. . . . . T . C	A . . . . . A . T . . . . . T T . . C . T . A . . A . . . . . A A T A . . . . . A C A . . . . A .
Rs-Tc	. . . . . T . C	A . . . . . A . T . . . . . T T . . C . T . A . . A . . . . . A A T A . . . . . A C A . . . . A .
Rf	. . . . G T . . . . . G .	A . . . . . A . T . . . . . T T . . C . T . A . . A . . . . . A A T G . . . . . A C A C . G . A .
Rl-Cdm	. . . A . . . C . T . T G	A . . . C . . . . A . T . . . A T . . . . A A . . . . A C . . . G . . G . . . . A . . . C
Rl-Via	. . . A . . . C . T . T G	A . . . C . . . . A . T . A . A T . . . . A A . . . . . C . . . G . . G . . . . A . . . C
Rlc-Cor	. . . A . . . C . . A T .	A . . . . . G . . . . A . T . . . . . T . . . A A . . . . T G . . . . A . C . .
Rsp-Bag	. . . . . C . A . . .	A . . . . A . . . . A . T . . . . . A . . . . G . . . .
Rsp-Dom	. . . . . C . A . . .	A . . . . A . . . . A . T . . . . . A . . . . G . . . .
Rsp-Gom	. . . . . C . A . . .	A . . . . . C . . . . A T T . . . . . C . . . . A . . . . G . . .
Rsp-Sa	. . . . . C . A . . .	A . . . . . C . . . . A T T . . . . . C . . . . A . . . . G . . .
Rbk-Dio	. . . . . C . A . . G	A . . G . . . C . A . . . . . . C . . . . T . . A . . . . T . T . . . G . . . .
Rspe	. . . . . C . A . . G	A A C . . . . . C . . . T . . G . . T . . T . . . A . . . . G . . . C A . T . . C . . . .
Rcly	C T G . . . . C . A . . .	A . . G . . . C T A . . . . . C . . . . T . . A . . . . T . . A . . G C . . . .
Rl-Kon	. . . . . C . A . . .	A . . G . . . . . A . . . . . . C . . . . T . . A . . . . T . . G . . G . . . . C . . . .
Rl-Ank	. . . . . C . A . . .	A . . G . . . C . A . . . . . . C . . . . T C . . A . T . . . . T A . G . . G . . A . . . .

Colony	3 3 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7
	8 9 0 0 1 1 1 2 2 2 3 5 5 5 6 6 7 7 7 8 8 8 8 9 0 0 1 1 2 2 2 2 3 3 4 4 5 5 5 6 7 7 7 7 8 0 0 1 1 2 2 3 4 4 4 4 6 6 7 7 7 9 0 0 0 1 1 1 3 3 4
	9 7 4 7 6 7 9 2 8 9 1 0 2 9 1 4 0 3 9 2 3 6 8 1 0 6 0 5 1 4 7 8 0 3 3 5 1 4 5 0 2 5 6 8 4 0 8 4 7 0 3 8 1 2 4 7 2 5 1 4 5 7 1 4 8 0 1 9 2 8 3
Rb-Bez	G G G C A G T T T T G G T T G T T G T G T C G T G T A C G G T T A A G T T A T T G G G T T A T A T T G G T A T T G C T A T A T T T A G T T G G
Rb-Vid	. . . . G . . . . .
Rg-For	. . A . G . . . C . . . . . A . . . . . T . . . . C . . . . . A . C . . G . . . . .
Rg-Ad	. . A . G . . . . A . . . . . T . . . . C . . . . . A . C . . G . . . . .
Rs-Ole	A . A T . . . . . C . A C . . . . . T A . . . . . G . . G . . . . . A . . . . . G . C . . C . . . A T . G . G . . . G . C . A .
Rs-Tc	A . A T . . . . . C . A C . . . . . T A . . . . . G . . G . . . . . A . . . . . G . C . . C . . . A T . G . G . . . G . C . A .
Rf	A . . T . T . . . . . C . A . . . . . T A . . . . . A . G . . . . . A . A . . . C G . C . A . . . . . T . G . G . . . G A C . A .
Rl-Cdm	. . . T G . . . . . A . . . C . . . . . T . C . . . . A . . . . A . A . . . . G . . A . . . . G . . G . .
Rl-Via	. . . T G . . . . . A . . . C . . . . . T . C . . . . A . . . . A . A . . . . G . . A . . . . G . . . . .
Rlc-Cor	A . . T G . . . . A C . . . . . T . C . . . . A . . . . . A . . . . . A . . . C . . G . . . . G . . . G . . . A A
Rsp-Bag	. A . T G . . . . C . . . . A C A . . A C T . C A . G . . A C . . . . A . . . . A A . . . . . G . . . . . G . . A . C G . G . . C . . . . .
Rsp-Dom	. A . T G . . . . C . . . . A C A . . A C T . C . . G . . . C . . . . A . . . . A A . . . . . G . . . . . G . . A . C G . G . . C . . . . .
Rsp-Gom	A A . T G . . . . C . . . . A C A . . A C T . C . . G . . . C . . . . A . . . . A A . . . . . G . . . A . G . . . . C G . G . . C . . . . .
Rsp-Sa	A A . T G . . . . C . . . . A C A . . A C T . C . . G . . . C . . . . A . . . . A A . . . . . G . . . A . G . . . . C G . G . . C . . . . .
Rbk-Dio	A . A T G . . . . C . . . C A . A A . . C T . . . . G T . . C . G . . . . C . . A A . . . . . G . . . . . G . C A . . G . G C . C G . . . . .
Rspe	A . . T G . . C . . . . . . A C . . C . C T . . . . . G . . . . . C C A A . . . G . G . . . . . C . . . G A G . . C G . . . A A
Rcly	A . A T G . . . . . A . C C A . A A . . C T . . . C G . . . . . G . . . . . . A A . . . . . G . . . . . G C C A . . G . G C C C G . . . . .
Rl-Kon	A . A T G . . . . . C C A . A . . . C T . . . . G T . . . C G . . . C . . . T A . C . . . G . . . . . G . C A . C G . G C . C G . . . . .
Rl-Ank	A . . T G . C . . . . . C C . . A A . . C T . . . . G . . . C . G . . . C . . . A A . . C . . . G . . . . G . C . . . G . G C . C G . . . . .

Rb-Bez	ITS2	Haplotypes	
	1 1 1 1 1 2 2 2 2 2 2 2 3 3 3 3 3 3 3		
	7 0 0 0 5 7 0 4 4 5 5 7 7 0 0 0 0 3 4 5		
	6 1 3 4 5 5 9 4 5 6 9 4 6 1 3 5 8 8 3 6	16S-ND1	ITS2
Rb-Bez	T T A A G A T A T G A G G G G A C C T T	1	1
Rb-Vid	. . . . .	2	1
Rg-For	C G . C . T	3	2
Rg-Ad	C G . C . T	4	2
Rs-Ole	C G . C T . C T . . T . . . . . A . C .	5	3
Rs-Tc	C G . C T . C T . . T . . . . . A . C .	5	3
Rf	C G . C T . C T . . T . . . . . A . C .	6	3
Rl-Cdm	C G G T . . . . . C	7	4
Rl-Via	C G G T . . . . . C	8	4
Rlc-Cor	C G . C	9	5
Rsp-Bag	C G . C . . C . . T . C . . C	10	6
Rsp-Dom	C G . C . . C . . T . C . . C	11	6
Rsp-Gom	C G . C . . C . . K . C . . C	12	6
Rsp-Sa	C G . C . . C . . T . C . . C	12	6
Rbk-Dio	C G . . . . C . A . . . . . T . . . A	13	7
Rspe	C G . C A . C . . . T . . . A T	14	8
Rcly	C G . . . . C . A . . . . . T . . . A	15	7
Rl-Kon	C G . . . . C . A . . . . . T . G . A	16	9
Rl-Ank	C G . . . . C . A . . . T . . T . . . A	17	10

the best fit for our data set. Rate matrix parameters were estimated on a NJ tree (PAUP rate matrix: abaaea; $a = 1$, $b = 17.43$, and $e = 11.89$). Base frequencies were $A = 0.19$, $C = 0.11$, $G = 0.21$, and $T = 0.49$. The shape of gamma distribution was 0.1922 with four rate categories. Results of ML analysis are shown in Fig. 2b.

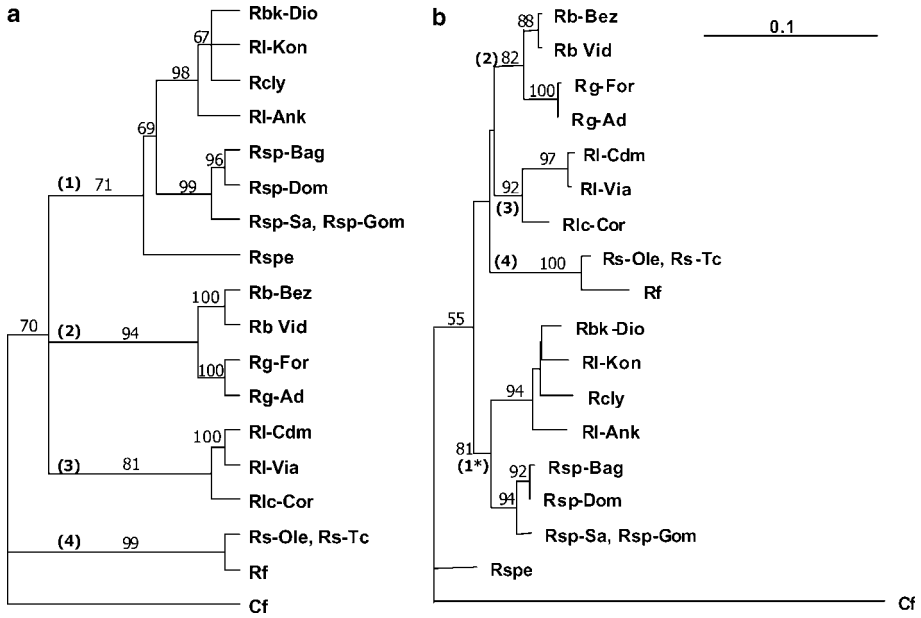

Fig. 2. Trees based on 16S-ND1 sequences (667 bp). (a) Cladogram of the strict consensus tree from four equally most parsimonious trees of 276 steps (CI: 0.701 and RI: 0.722). (b) Maximum likelihood phylogram ($-\ln L = 2212.343$). Numbers above branches indicate bootstrap support percentage over 50% in 2000 (MP) or 100 (ML) pseudo-replicates. Number in parentheses refer to the groups described in Results. Groups (1) and (1*) differ for the position of *Reticulitermes speratus*.

Terminal branching was consistent with the MP tree except that the position of *R. speratus* was subtracted from group 1. All terminal branches were well supported (bootstrap values $\geqslant 70$). Deep branching topology differed between MP and ML trees: the group 4 (*R. santonensis* and *R. flavipes*) was basal to the other *Reticulitermes* species in the MP tree, and showed a not resolved position in the ML tree (bootstrap values <50).

The bootstrap consensus NJ tree was similar to the tree obtained by ML analysis. The differences were a strongly supported node (80) for the monophyletic clade including groups (2) and (3), and the position of *R. speratus* that was basal to the group (1*) although the node showed a low bootstrap support (63) (data not shown).

In all analyses *R. sp. nov.* samples from Italy and France clustered together, showing an affinity with *Reticulitermes* termites collected in the eastern Mediterranean area. *R. speratus* from Japan clustered basal to this group only in the MP and NJ analyses.

Two hypotheses were tested using ML trees. The first hypothesis (CONSTRAINT 2) implied that *R. sp. nov.* and *R. speratus* were monophyletic based on the assumption that *R. sp. nov.* was accidentally introduced from Japanese populations (Marini and Mantovani, 2002). The second hypothesis (CONSTRAINT 1) was that *R. sp. nov.*, *R. clypeatus*, *R. l.*-Turkey, and *R. balkanensis* constituted a monophyletic group based on the assumption of a common ancestor in the eastern area. Using ML ratio tests based on parametric bootstrap, the null hypothesis that *R. sp. nov.*, *R. clypeatus*, *R. l.*-Turkey, and *R. balkanensis* (CONSTRAINT 1) formed a monophyletic group could not be rejected but the hypothesis that *R. sp. nov.* and *R. speratus* were monophyletic (CONSTRAINT 2) was rejected ($p < 0.01$) (Fig. 3). Since these findings indicate that the monophyletic *R. sp. nov. R. speratus* group did not result from stochastic variations in ML analysis, the hypothesis that *R. sp. nov.* populations originated from *R. speratus* cannot be accepted.

Pairwise Tamura–Nei distances showed a more restricted range of values for termites in the western European area (except *R. santonensis*) than termites in eastern area (*R. balkanensis*, *R. clypeatus*, *R. l.*-Turkey). Within the western area distances ranged from 0.0015 (Rg-For vs Rg-Ad) to 0.0046 (Rl-Cdm vs Rl-Via) at the intraspecific level and from 0.0322 (*R. banyulensis* vs *R. grassei*) to 0.1010 (*R. sp. nov.* vs *R. lucifugus*) at the interspecific level. In the eastern area distances ranged from 0.0276 (Rl-Kon vs Rbk-Dio) to 0.0481 (Rl-Ank vs Rcly). *R. balkanensis* showed the lowest divergence with *R. sp. nov.* (0.0635).

3.1.2. ITS2 complete sequence

We obtained the complete sequences of the ribosomal internal transcribed spacer (ITS2, 382 bp). The 10 haplotypes scored (Table 3) showed only 14 variable sites (3.7%), demonstrating little sequence variability compared to the partial 16S-ND1 region. Interestingly, we observed a same haplotype for *Reticulitermes banyulensis* (Rb-Bez, Rb-Vid), for *R. grassei* (Rg-For, Rg-Ad), for *R. santonensis* and *R. flavipes* (Rs-Ole, Rs-Tc,

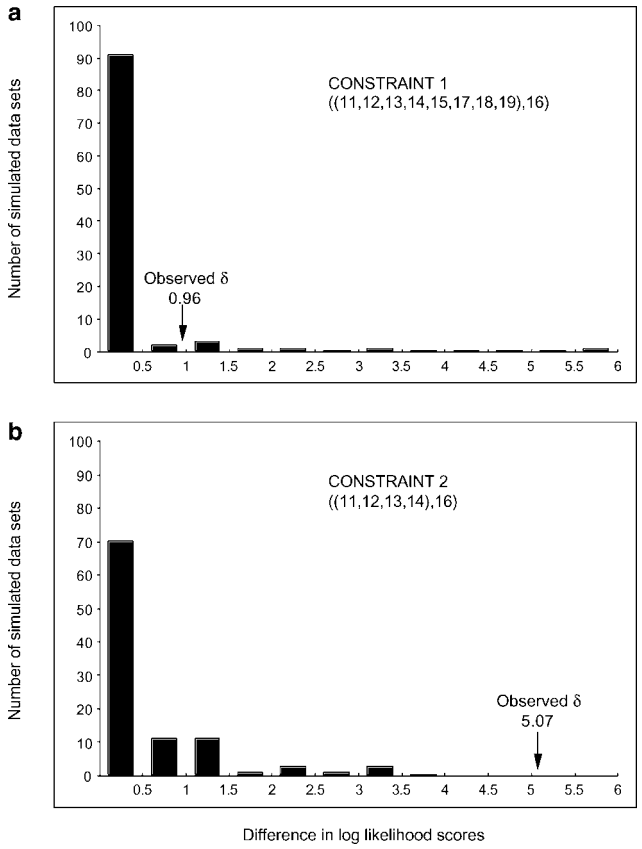

Fig. 3. Results of the parametric bootstrap analysis. Differences in log likelihood scores between the constrained (null hypothesis) and unconstrained trees for 100 replications were recorded to obtain the expected distribution under the null model. (a) Since seven simulated differences fall below the difference for the observed data ($\delta = 0.96$), the null hypothesis cannot be rejected. (b) All 100 sampled differences fall below 3.5, whereas the observed score was 5.07. We conclude that the alternative hypothesis (CONSTRAINT 2) can be rejected at $p < 0.01$. See Table 1 for the reference numbers used in the constraints.

Rf), for *R. lucifugus* (Rl-Cdm, Rl-Via), for *R. balkanensis* from Greece and *R. clypeatus* from Israel (Rbk-Dio, Rcly), and for the four *R. sp. nov.* samples (Rsp-Bag, Rsp-Dom, Rsp-Gom, Rsp-Sa).

Construction of phylogenetic trees using NJ method did not fully resolve the relationships between the ten haplotypes. The bootstrap analysis strongly supported the branch including the haplotypes 7, 9, and 10 corresponding to the samples from the Eastern Mediterranean area (data not shown). ML analysis produced similar results (data not shown), while MP analysis resulted in 120 most-parsimonious trees. In the last case the strict consensus tree derived from these trees did not show any clear relationships between clades (data not shown).

3.2. Molecular clock

The molecular clock hypothesis for the ND1 gene (535 bp) could not be rejected. The likelihood of the ND1 tree was $-\ln L = 1877.3070$ with molecular clock enforced versus $-\ln L = 1868.6420$ without the molecular clock enforced. This difference was not significant (p value $= 0.30$; df $= 15$). This finding suggested that lineages in our data set evolved at the same rate. Thus the ND1 clock could be used to estimate divergence time between lineages, along with others genes showing a molecular-clock behavior. In fact, several protein clocks should be used to estimate divergence time accurately (Ayala, 1997).

4. Discussion

4.1. Phylogeny and distribution of Reticulitermes termites

All terminal clades in this study were well supported, according to previous studies demonstrating important phenotypic differences in cuticular hydrocarbon profiles (Bagnères et al., 1988, 1991) and soldier-gland secretion composition (Bagnères et al., 1990; Parton et al., 1981; Quintana et al., 2003) between the different *Reticulitermes* species.

Reticulitermes banyulensis and *R. grassei* which are found in France and Spain and live sympatrically in eastern Spain appeared to be closely related (Clément et al., 2001). This finding is in agreement with the hypothesis that the two species evolved from a common ancestor that survived the glacial period in a refugium in southern Spain. The area where the *R. banyulensis* sample was collected in southern France, Rb-Vid, currently represents the easternmost limit of the species distribution range. In that collection site *R. banyulensis* lives sympatrically with *R. lucifugus*.

The Italian species *R. lucifugus* and Corsican subspecies *R. l. corsicus* were placed in a well supported clade distinct from that of the *R. sp. nov.* samples collected in Italy. Thus, *R. sp. nov*. can not be classified as *R. lucifugus*.

Reticulitermes santonensis from France and *R. flavipes* from USA clustered together in phylogenetic trees obtained with 16S-ND1 sequences. However, they formed a clade basal to the other *Reticulitermes* species in the MP tree and were included in the polytomy in the ML and NJ trees. Interestingly, *R. santonensis* and *R. flavipes* had the same consensus sequences for the ITS2 region, as observed by Jenkins et al., 2001. The limited distribution range of *R. santonensis* in Europe (Vieau, 2001) and the wide distribution range of *R. flavipes* in North-America are in agreement with the hypothesis that the species could be native to North America and was accidentally introduced into Europe (Bagnères et al., 1990).

Turkish *R. lucifugus* (R.l.-Kon, R.l.-Ank), *R. balkanensis* (Greece) and *R. clypeatus* (Israel) formed a single clade well supported. Moreover, *R. balkanensis*

and *R. clypeatus* shared a same ITS2 haplotype. Similar results were obtained by Austin et al. (2002) with the mitochondrial DNA COII region. These results could be explained by a common origin of the termite populations during glacial movements (see below for a more detailed explanation). The Authors suggest that the taxonomy in this area should be considered carefully: further studies could detect some homonymies.

4.2. Origin of *R. sp. nov.*

The four samples of *R. sp. nov*. studied formed a single clade when 16S-ND1 sequences were analyzed. Moreover they had the same sequence for the ITS2 region. This suggests that the *R. sp. nov*. samples belong to the same taxonomic group. Prior to the recent discovery of a natural site in southern France (Sophia Antipolis), all samples of *R. sp. nov*. had been collected in urban settings. Such a distribution suggested that *R. sp. nov*. might be a non-western European species imported by man. However, results obtained in this study by non-parametric bootstrap strongly support a relationship between *R. sp. nov*. and eastern Mediterranean termites including *R. clypeatus* from Israel, *R. lucifugus* from Turkey and *R. balkanensis* from the Balkans. The species are characterized morphologically by the presence of a similar post-clypeus (Clément et al., 2001). The hypothesis of a relationship with *R. speratus* was rejected by parametric bootstrap.

The Quaternary cold periods are thought to have influenced distribution ranges of both animal and plant life in the Mediterranean area. Several authors have attributed the dispersion patterns shared by many taxa to these periods (De Jong, 1998; Taberlet et al., 1998). Successive contraction and expansion of distribution ranges during these periods may have resulted in a loss of genetic diversity in northern species (bottlenecks). Similarity between species in different geographical locations could be linked to postglacial colonization routes between Balkan refugia and northern regions. The Alps could act as a barrier preventing the northward progression. *R. sp. nov*. may have reached southern France by crossing Italy and passing along the Mediterranean coast. Notwithstanding the fact that most *R. sp. nov*. samples have been collected in urban sites, the geographical location of collection sites is in agreement with the hypothesis of postglacial colonization. These termites may have been introduced into urban zones from neighboring regions. The small number of *R. sp. nov*. sites discovered up to now ($n = 15$; Mira, Salsomaggiore, Bagnacavallo, S. Agata sul Santerno, Galatina, Ravenna in Italy and Domène-38, Grenoble-38, Château Gombert-13, La Ciotat-13, St-Cyr-Les-Lecques-83, St. Laurent du Var-06, Sophia Antipolis-06, St. Paul de Vence-06, Roquebrune Cap Martin-06, in France) could be linked either to the recent availability of new technology for species identification or to successive contraction and expansion of distribution range during Quaternary cold periods. In the latter case, remnants of present-day sites would be left over from wider distribution of the past.

This work demonstrated the value of the use of both nuclear and mitochondrial markers to assess phylogenetic patterns and taxonomic status. Both markers agreed in defining the specific status for the *R. sp. nov*. populations, and provided evidence to identify the area from which *R. sp. nov*. is likely to have originated from. The genetic distance between *R. sp. nov*. and species in eastern Europe are of the same magnitude as interspecific distances observed in western Europe. Several features may have contributed to the development of reproductive isolation mechanisms in *R. sp. nov*. (Clément et al., 2001) and to its specific differentiation status. Description of the new species will be published elsewhere (Bagnères and Uva, unpublished data). Samples will be sent to the Museum d'Histoire Naturelle in Paris, France, as voucher specimens.

Future studies will demand that *Reticulitermes* termites from other countries in the eastern-Mediterranean area be examined. However, investigators in that part of Europe still use the old Rossi classification system (1792) which includes only one *R. lucifugus* species. A clear classification of the genus will be needed to study the mechanisms underlying speciation in the western Palearctic region.

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A Comparative Genetic Analysis of the Subterranean Termite Genus *Reticulitermes* (Isoptera: Rhinotermitidae)

JAMES W. AUSTIN,[1, 2] ALLEN L. SZALANSKI,[3] PAOLO UVA,[4] ANNE-GENEVIEVE BAGNÈRES[2] AND AYKUT KENCE[1]

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ABSTRACT DNA sequencing analysis of the mitochondrial DNA cytochrome oxidase II (COII) region was used to examine genetic variation in the termite genus *Reticulitermes* Holmgren. We examined 21 species and subspecies from three continents. Sequencing of a 677-bp region of a 780-bp amplicon from 41 individuals and from 17 sequences obtained from GenBank revealed 221 polymorphic sites within the genus. Tajima–Nei distances from species ranged from 0.9 to 12.7%, and parsimony and maximum likelihood analysis revealed several clades within the genus. *Reticulitermes flavipes* (Kollar) formed a distinct clade along with *R. santonensis* De Feytaud. European *R. lucifugus* (Rossi) formed a distinct clade with *R. banyulensis* (Béziers). Turkish *R. lucifugus* was distinct relative to European *R. lucifugus*, and along with *R. clypeatus* Lash from Israel formed a sister group with *R. balkanensis* Clément from Greece. This study provides support for the separation of Turkish *R. lucifugus* from European members of the species. This mitochondrial DNA marker was also able to identify several *Reticulitermes* specimens from Oklahoma, Texas, Missouri, and South Korea to *R. flavipes*, *R. hageni* Banks, *R. virginicus* (Banks), and *R. speratus* Shimizu.

KEY WORDS COII, DNA sequence, genetic variation, population genetics, *Reticulitermes*, termite

SPECIES OF THE genera *Reticulitermes* Holmgren (Isoptera: Rhinotermitidae) are the major termite pests infesting wooden structures in the United States and other countries. It has been estimated that more than $1.5 billion is spent annually for termite control in the United States, of which 80% is spent to control subterranean termites (Su 1993). A breakdown of damage caused by termite species reveals that the five principal subterranean termite species in the United States are *Reticulitermes flavipes* (Kollar), *Reticulitermes virginicus* (Banks), *Reticulitermes hesperus* (Banks), *Reticulitermes tibialis* (Banks), and *Coptotermes formosanus* (Shiraki). Ninety percent of the termite control industry involves these five subterranean termite species (Forschler and Lewis 1997).

Reticulitermes spp. are the most abundant naturally residing termites in Europe, with six described phenotypes that have been identified on the basis of morphological, chemical (cuticular hydrocarbons and soldier defensive secretions), and molecular (enzymatic alleles and mitochondrial ND1 sequence) characters (Clément et al. 2001). *Reticulitermes* spp. in Europe are known pests of urban structures and frequently pose threats to various agricultural crops.

Unlike in Europe, the distribution of termites and their subsequent impact as an economic pest in developing countries such as Turkey have not been reported. The Mediterranean termite *Reticulitermes lucifugus* (Rossi) was first described by Weber (1954) as occurring in the Zubair desert region, Iraq, and it has been hypothesized by Weidner (1972) that *R. lucifugus* may in fact belong to *R. clypeatus* Lash. *R. lucifugus* (Rossi, 1792) has been the only documented species of the genera *Reticulitermes* to occur in Turkey (Bodenheimer 1958, Weidner 1972, Lodos 1982, Karaat and Göven 1983).

Information on how genetic variation is partitioned within populations and among termite species can be useful for determining the extent of gene flow, and for developing molecular diagnostics for identifying species. Previous studies (Jenkins et al. 1998, 2001; Marini and Mantovani 2002) have focused on *Reticulitermes* spp. from the southeastern United States or Western Europe, but have not included populations from other areas of the world where these termites occur.

The cytochrome oxidase II (COII) region of the mitochondrial DNA (mtDNA) genome has proved useful for the phylogenetic relationship of termites (Miura et al. 1998; Jenkins et al. 1999, 2001; Lo et al. 2000). We investigated the phylogenetic relationships among *Reticulitermes* spp. from three continents and determined the amount of genetic differentiation among several disjunct *R. lucifugus* populations.

[1] Department of Biology, Middle East Technical University, Ankara, Turkey.
[2] Current address: Department of Entomology, University of Arkansas, Fayetteville, AR 72701.
[3] Department of Entomology, University of Arkansas, Fayetteville, AR 72701 (e-mail: aszalan@uark.edu).
[4] C.N.R.S.–I.R.B.I., Faculté des Sciences, Tours, France.

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Table 1. Termite collection data

Species	Collection Site	Country	GenBank
R. balkanensis	Shinias	Greece	AF525318
R. banyulensis	Béziers	France	AF525319
R. chinensis	Beijing	China	AB050705
R. clypeatus	Ben Shemen	Israel	AF525320
R. flaviceps	Tokunoshima	Japan	AB050708
R. flavipes	Grand Bahamas	Bahamas	AF525322
	Toronto, ON	Canada	AF525326
	Hamburg strain "A"	Germany	AF525324
	Hamburg strain "B"	Germany	AF525323
	Alachua Co., FL	USA	AF525321
	Lincoln, NE	USA	AF525325
	Sapelo Island, GA	USA	AF107484
R. grassei	Charente	France	AF525327
		Italy	AF291744
R. guangzhouensis	Guangzhou	China	AB050709
R. hageni	Barnesville, GA	USA	AF107486
	Cumberland Island, GA	USA	AF525328
R. hesperus	Los Angeles, CA	USA	AF525329
R. labralis		China	AB050711
R. lucifugus	Corsica	France	AF525332
	Narbonne	France	AB050707
	Sardegna	Italy	AF291730
	Castel	Italy	AF291724
	Chieti	Italy	AF291738
	Palermo	Italy	AF291741
	Patanella	Italy	AF525341
	Dış Kapı, (Ankara)	Turkey	AF525333
	Antayla	Turkey	AF525330
	Fethiye	Turkey	AF525334
	K. Maraş	Turkey	AF525338
	Kaş	Turkey	AF525336
	Konya	Turkey	AF525337
	Izmir	Turkey	AF525335
	Mersin	Turkey	AF525339
	Muğla	Turkey	AF525340
R. 1. corcicus	Corsica	France	AF525331
R. n. sp.	Catalina Island, CA	USA	AF525342
R. perilabralis		China	AB050710
R. santonensis	Charente	France	AF525343
		France	AF262607
R. sp.	Conway, AR	USA	AF525349
	Columbia, MO	USA	AF525348
	Oklahoma City, OK	USA	AF525353
	Arlington, TX	USA	AF525345
	Bryan, TX	USA	AF525346
	College Station, TX	USA	AF525347
	Ft. Worth, TX	USA	AF525351
	Mansfield, TX	USA	AF525352
	Domene	France	AF525350
	Taejon	S. Korea	AF525354
R. speratus	Mito City	Japan	AF525344
		Japan	AB005584
R. tibialis	Cochise Co., AZ	USA	AF525355
R. virginicus	Pinellas Co., FL	USA	AF525356
	Roanoke, VA	USA	AF525357
Coptotermes formosanus	Galveston, TX	USA	AF525317
Heterotermes tenuior	Borneo Island	Indonesia	AB050714
Panesthia cribrata			AF220580

GenBank DNA sequences from this study are italicized.
R., Reticulitermes.

Materials and Methods

Termites were collected from various locations in North America, Europe, and Asia and preserved in 70% ethanol (Table 1). For morphological identification of *R. lucifugus*, we used (1) a modified biometric analysis method using winged, immature and neotenic termites belonging to French, Turkish, and American populations of the genus *Reticulitermes* (Clemént 1978, 1979a); and (2) discriminate function analysis (Mayr and Ashlock 1991) of worker and soldier castes from other available *R. lucifugus* specimens previously collected. *R. lucifugus* from Turkey was validated using a cuticular hydrocarbon analysis by gas chromatography-mass spectrometry (GC-MS) and quantified

Table 2. Tajima–Nei pairwise distances among 19 *Reticulitermes* taxa

No.	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*R. ampliceps* CHN	–	8.8	8.5	3.5	8.5	11.2	10.4	7.0	8.8	8.2	7.5	6.6	8.7	11.0	7.8	10.3	6.8	10.1	9.5
2	*R. balkanensis* Shinias, GRC		–	8.0	8.6	4.5	10.4	9.3	8.9	6.7	7.0	9.1	4.0	8.7	9.9	9.4	9.9	8.0	6.8	7.3
3	*R. banyulensis* Beziers, FRA			–	8.0	8.5	9.4	7.4	7.5	5.5	7.2	7.5	7.0	6.0	8.7	7.8	8.5	8.9	8.2	7.7
4	*R. chinensis* CHN				–	8.8	10.2	10.4	6.1	9.5	7.3	7.0	6.6	8.9	10.4	7.0	9.4	6.3	9.8	8.4
5	*R. clypeatus* Ben Shemen, ISR					–	10.5	10.1	8.0	6.7	8.2	8.9	3.7	9.1	9.0	8.9	9.8	8.7	7.8	7.9
6	*R. flavipes* NE, USA						–	11.6	8.8	8.2	9.4	9.0	9.9	11.4	12.7	9.2	1.7	12.7	9.9	9.8
7	*R. grassei* Charente, FRA							–	9.9	8.7	7.8	10.8	8.5	8.3	6.8	10.8	10.6	9.3	10.3	8.0
8	*R. guangzhouensis* Guangzhou, CHN								–	8.1	7.7	1.7	7.3	8.9	10.1	1.7	8.0	8.0	8.2	8.1
9	*R. hageni* GA, USA									–	5.7	8.1	6.2	7.5	8.9	8.4	7.5	9.1	5.0	8.3
10	*R. hesperus* CA, USA										–	8.9	6.5	7.9	9.0	8.6	8.5	7.1	7.9	7.2
11	*R. labralis* CHN											–	7.5	8.7	10.7	0.9	8.2	8.7	8.0	8.6
12	*R. lucifugus* Antalya, TUR												–	7.5	7.6	7.8	9.0	7.5	6.5	6.5
13	*R. lucifugus* Corsica, FRA													–	11.7	8.7	10.1	9.0	9.0	8.6
14	*R. n. sp.* CA, USA														–	10.5	11.2	10.5	10.3	7.1
15	*R. perilabralis* CHN															–	8.3	8.9	8.4	8.7
16	*R. santonensis* Charente, FRA																–	11.8	9.1	8.4
17	*R. speratus* Mito City, JPN																	–	10.5	8.0
18	*R. virginicus* FL. USA																		–	7.4
19	*R. tibialis* AZ, USA																			–

by gas chromatography using an internal standard for each caste and all colonies (PU, unpublished data). GC-MS was only used for the Turkish samples because the samples must be preserved in pure pentane, and at least 20–30 individuals are needed for each sample. Identification of other *Reticulitermes* spp. collected in this study was done using keys by Weidner (1959, 1960, 1972), Krishna and Weesner (1969), Clément (1978), Scheffrahn and Su (1994), and Donovan et al. (2000).

Voucher specimens, preserved in 70% ethanol, are maintained at the Arthropod Museum, Department of Entomology, University of Arkansas, Fayetteville.

Alcohol-preserved specimens were allowed to dry on filter paper, and DNA was extracted from individual heads using the Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN). Extracted DNA was resuspended in 50 μl of Tris:EDTA and stored at –20°C. Polymerase chain reaction (PCR) was conducted using the primers TL2-J-3037 (5′-ATGGCAGATTAGTGCAATGG-3′) designed by Liu and Beckenbach (1992) and described by Simon et al. (1994) and Miura et al. (1998), and primer TK-N-3785 (5′-GTTTAAGAGACCAGTACTTG-3′) from Simon et al. (1994). These primers amplify a 3′ portion of the mtDNA COI gene, tRNA-Leu, and a 5′ section of the COII gene. PCR reactions were conducted using 1 μl of the extracted DNA (Szalanski et al. 2000), with a profile consisting of 35 cycles of 94°C for 45 s, 46°C for 45 s, and 72°C for 60 s. Amplified DNA from individual termites was purified and concentrated using Microcon-PCR Filter Units (Millipore, Bedford, MA).

Samples were sent to the University of Arkansas DNA Sequencing Facility (Fayetteville) for direct sequencing in both directions using an ABI Prism 377 DNA sequencer. GenBank accession numbers for the termites subjected to DNA sequencing in this study are AF525317 to AF525357 (Table 1. The accession numbers for the DNA sequences of additional *Reticulitermes* spp. termites obtained from GenBank are also provided in Table 1.

The distance matrix option of PAUP* 4.0b8 (Swofford 2001) was used to calculate genetic distances according to the Kimura 2-parameter model (Kimura 1980) of sequence evolution. Mitochondrial DNA COII sequences from the Australian wood feeding cockroach, *Panesthia cribrata* Saussure (GenBank AF220580); Formosan termites, *Coptotermes formosanus* Shiraki and *Heterotermes tenuior* (Haviland) (GenBank AB050714) were added to the *Reticulitermes* DNA sequences to act as outgroup taxa. DNA sequences were aligned using the PILEUP program in GCG (Genetics Computer Group, Madison, WI) and adjusted manually. Maximum-likelihood and unweighted parsimony analyses on the alignments were conducted using PAUP*. Gaps were treated as missing data. The reliability of trees was tested with a bootstrap test (Felsenstein 1985). Parsimony bootstrap analysis included 1,000 resamplings using the Branch and Bound algorithm of PAUP*. For maximum-likelihood analysis, the default likelihood parameter settings were used (HKY85 6-parameter model of nucleotide substitution, empirical base frequencies, and transition/transversion ratio set to 2:1). These parameters were used to carry out a heuristic search using PAUP*, using either the single most parsimonious tree as the starting tree or step-wise addition.

Results

DNA sequencing of the amplicon revealed that it averaged 780 bp in size. To facilitate genetic comparisons with existing GenBank DNA sequences, 103 bp from the 5′ end of the amplicon was excluded and the remaining 677 bp COII portion was used. The average base frequencies were A = 0.39, C = 0.23, G = 0.14, and T = 0.24. The mtDNA COII *Reticulitermes* sequences were aligned using *P. cribrata*, *C. formosanus*, and *H. tenuior*, as the outgroup taxa. The aligned DNA data matrix, including the outgroup taxa (available at TreeBASE, http://www.treebase.org, study accession

Table 3. Tajima–Nei pairwise distances in *Reticulitermes lucifugus* and *R. grassei*

No.	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*R. lucifugus* Antalya, TUR	–	8.4	8.5	7.5	3.5	2.9	4.2	3.5	2.3	1.2	3.5	4.6	7.1	8.0	8.7	6.6	7.5	6.5
2	*R. l.* Castel, ITA		–	1.1	2.1	7.8	10.0	8.2	9.0	8.5	8.6	9.1	9.8	6.0	4.0	0.3	3.4	1.2	5.8
3	*R. l.* Chieti, ITA			–	3.2	8.0	10.1	8.4	9.2	8.7	8.4	9.2	9.9	5.8	3.5	1.4	3.2	1.8	5.6
4	*R. l.* Corsica, FRA				–	7.3	9.4	8.0	8.5	8.0	8.0	8.5	8.8	6.1	6.0	2.4	4.7	2.1	6.1
5	*R. l.* Ankara–Dış Kapı, TUR					–	5.0	1.2	4.0	4.0	3.9	4.0	4.2	7.0	7.3	7.8	6.0	7.7	6.1
6	*R. l.* Fethiye, TUR						–	5.3	5.0	1.5	2.6	5.0	5.8	9.2	9.6	10.3	8.2	9.4	8.2
7	*R. l.* Izmir, TUR							–	4.0	4.7	4.2	4.3	3.5	7.6	7.5	8.2	6.3	8.0	6.8
8	*R. l.* K. Maras, TUR								–	4.0	3.7	0.6	4.5	7.5	8.0	9.4	7.5	9.2	7.6
9	*R. l.* Kas, TUR									–	2.0	4.0	5.5	8.0	8.2	8.9	6.8	8.4	7.0
10	*R. l.* Konya, TUR										–	3.5	5.0	7.7	8.2	8.9	7.0	8.0	7.1
11	*R. l.* Mersin, TUR											–	4.8	7.5	8.2	9.4	7.5	9.2	7.6
12	*R. l.* Muğla, TUR												–	8.1	8.7	9.8	7.8	9.2	7.6
13	*R. l.* Narbonne, FRA													–	6.0	6.3	5.0	5.6	4.7
14	*R. l.* Palermo, ITA														–	4.3	3.1	4.5	5.5
15	*R. l.* Patanella, ITA															–	3.7	1.5	6.1
16	*R. l.* Sardegna, ITA																–	3.7	4.2
17	*R. grassei*, FRA																	–	5.1
18	*R. grassei*, ITA																		–

number S769) resulted in a total of 677 characters. Of these characters, 332 (49%) were variable, and 208 (31%) were phylogenetically informative. Pairwise Tajima–Nei distances (Tajima and Nei 1984) among *Reticulitermes* taxa ranged from 0.9% between *R. labralis* and *R. perilabralis*, to 12.7% between *R. flavipes* to *Reticulitermes* n. sp. (Table 2). Within *R. lucifugus*, pairwise Tajima–Nei distances ranged from 0.3% between Castel and Patanella, Italy, to 10.3% between Fethiye, Turkey, and Patanella, Italy (Table 3).

This data set had only one most parsimonious tree (Fig. 1), (length = 1,003, CI = 0.44), as documented using the Branch and Bound search algorithm of PAUP*. Bootstrap analysis of the aligned *Reticuli-*

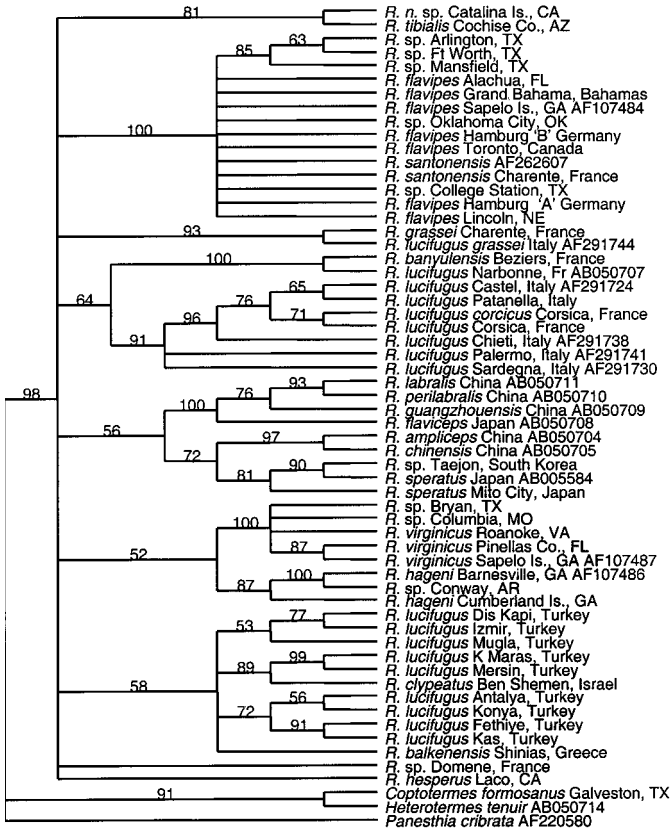

Fig. 1. Single most parsimonious tree during a branch and bound search using PAUP* (Swofford 2001). Bootstrap values for 1,000 replicates are listed above the branches supported at ≥50%.

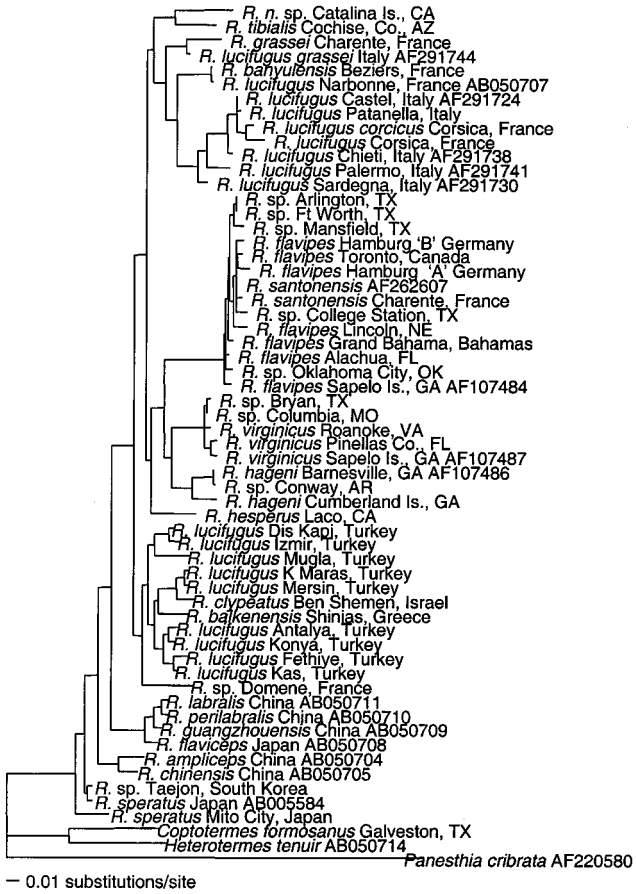

Fig. 2. Topology obtained by maximum-likelihood analysis based on the HKY85 model (see text). Log L = –5851.71969.

termes spp. and the outgroup taxa resulted in a consensus tree with several distinct branches. These distinct clades included: *R. flavipes* and *R. santonensis* De Feytaud; *R. banyulensis* (Béziers, France) and European *R. lucifugus; R. virginicus* and *R. hageni; R. clypeatus, R. balkanensis* and Turkish *R. lucifugus; Reticulitermes* n. sp. Scheffrahn, and *R. tibialis;* and the *Reticulitermes* spp. from China and Japan. *R. clypeatus* from Israel formed a distinct clade with *R. lucifugus* from K. Maraş and Mersin, Turkey. Based on the maximum parsimony analysis, we were unable to determine the relationship of *Reticulitermes* sp. Domene, France, and *R. hesperus* with the other taxa. Of the six *Reticulitermes* spp. from the United States that were not classified to species morphologically, four were members of the *R. lucifugus*/*R. santonensis* clade, and the other two belonged to the *R. virginicus* clade (Fig. 1).

Regardless of whether the starting tree was the most parsimonious tree or was obtained via step-wise addition, the maximum-likelihood search found only one tree (Fig. 2). The maximum-likelihood tree differed from the maximum-parsimony tree for two samples: the two *R. hageni* samples did not form a sister group with *R. virginicus;* and *Reticulitermes* sp. France formed a sister group with the samples from Turkey and Greece.

Discussion

In this study, a phylogenetic analysis of *Reticulitermes* belonging to 21 species and subspecies from three continents, based on the DNA sequence of a portion of the mitochondrial COII gene, is presented. The mtDNA COII marker was used to allow the incorporation of *Reticulitermes* spp. DNA sequences submitted to GenBank. Most of the inferred relationships had strong quantitative support as indicated by bootstrap analysis. The relationships among taxa inferred from maximum-parsimony and maximum-likelihood analyses were for the most part congruent with currently accepted groupings. For example, the grouping of *R. labralis, R. perilabralis* Ping and Xu, *R. guangzhouensis* Ping, *R. flaviceps* (Oshima), *R. ampliceps, R. chinensis* Snyder, and *R. speratus* (Kolbe) reflects a clear delimitation of eastern Asian *Reticulitermes* spp., when compared with their more western counterparts in Europe and North America (Fig. 1).

However, the addition of previously unsampled populations of *R. lucifugus* from Turkey in combina-

tion with various other unsampled populations from throughout the world resulted in some notable differences in their classification. The most notable difference was observed in the *lucifugus* complex, as previously described by Plateaux and Clément (1984), which included one ponto-Mediterranean probable species (*balkanensis*), one Mediterranean species (*lucifugus*) with a Corsican probable subspecies (*corsicus*), and an atlanto-Mediterranean species. The last two forms, Aquitanian (*grassei*) and Catalan (*banyulensis*), are naturally intersterile just like two different species, but they are connected by a range of interfertile Iberian forms that constitute a large zone of primary intergradation. Our results clustered *R. banyulensis* (Béziers) (with both *R. lucifugus* France, Narbonne and Corsica), *R. lucifugus corsicus* France (Corsica) and *R. lucifugus* Italy (Castel, Patanella, Palermo, and Sardinia) (Fig. 3).

The large zones of sympatric occupation of *Reticulitermes* spp., as previously described by Plateaux and Clément (1984), suggest the likelihood of hybridization in numerous instances (examples include, *R. grassei* and *R. santonensis* in southwestern France, and *R. banyulensis* and *R. grassei* stretching from northwestern Spain through Portugal to southeastern Spain on the Iberian peninsula). By modifying the breeding temperature, Clément (1979b) demonstrated that artificial constitution of heterospecific pairs allowed hybridization between sympatric *R. santonensis* and *R. lucifugus.* However, because these termites possessed different flagellates, suggesting they do not naturally intermingle, there was no support for natural hybridization to occur. Clément et al. (2001) suggest that this demonstrates the efficacy of the species' isolation mechanism between them. It might also demonstrate, with respect to *Reticulitermes* spp. in other locations, that large sympatric zones may (1) provide the framework for natural hybridization to occur or (2) be a function of environmental similarities that potentially induce clinal variations in *Reticulitermes* spp., where otherwise strong species isolation mechanisms (whether behavioral, chemical, chronological with respect to mating time, or genetic) are rendered inadequate to prevent hybridized mating.

More recent discoveries of *R. grassei* in southwestern England (Fig. 3), where it was previously believed that termites were incapable of persisting, support the concept that some isolation mechanisms may be rendered inactive in the presence of anthropogenic interference. The behavioral adaptation, whereby termites migrate to structures for warmth emitted from buildings in the winter months, permits the survival of the respective species outside of its believed normal habitation range. A similar scenario has been described for the occurrence of *R. flavipes* in Toronto, Canada, and Hamburg, Germany (Weidner 1970).

In our study, the clade containing *R. grassei* was clearly distinct from *R. lucifugus* in France and from *R. lucifugus* in Turkey (Fig. 1). These results are supported by phylogenetic analyses of DNA sequences of the mtDNA 16s rRNA gene and NADH dehydrogenase one genes, and GC-MS analyses of cuticular hydrocarbons (unpublished data). The clade containing *R. lucifugus* (France and Italy), *R. banyulensis* (which occurs in the southern Mediterranean coastal areas of France and Spain), and *R. lucifugus corcicus*, likewise formed a distinct group. Plateaux and Clément (1984) describe *R. banyulensis* as being intersterile, like a different species, but connected by a range of interfertile Iberian forms that constitute a large zone of primary intergradation. Although *R. lucifugus corcicus* is considered morphologically similar to certain populations of *R. grassei* (Clément 1982), in our study, *R. lucifugus corcicus* formed a sister clade with other *R. lucifugus* populations from France and Italy (Fig. 1). Our results suggest that *Reticulitermes* sp. from Domene France is either the result of hybridization of two sympatric species within the zones of their occurrence, or most probably an introduced *Reticulitermes* sp. from one of the neighboring countries or regions.

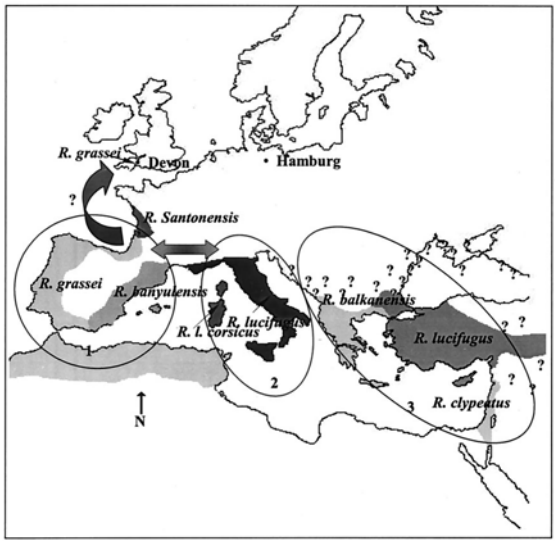

Fig. 3. Natural geographical distribution of *Reticulitermes* species in Europe. 1, the *R. grassei–R. banyulensis* group; 2, *R. lucifugus* in Italy; 3, the *R. balkanensis* GRE–*R. lucifugus* TUR–*R. clypeatus* ISR group. The arrow between groups 1 and 2 reflects the pairing of *R. banyulensis* with *R. lucifugus* Italy as was observed in the phylogenetic analysis.

Turkish *R. lucifugus* formed sister clades with *R. balkanensis* (Greece) and *R. clypeatus* (Israel) that were clearly distinct from French and Italian *R. lucifugus* (Figs. 1 and 2). It would appear that the differences observed from the common clade containing *R. balkanensis*, *R. lucifugus* (Turkey), and *R. clypeatus* (Israel) with taxa from more northerly locations support geographic isolation that is clinal in nature (Fig. 3). Weidner (1960) supports this hypothesis using morphological analysis of a limited number of samples. He concluded that termite morphology is influenced by geography. An increase in body size occurs in an east-to-west direction. The gula was broader at the northern edge of the circulation area (La Rochelle,

France, and Dojran, Macedonia) compared with a slimmer gula in the more southern areas (Madeira, Israel, and Iraq). Likewise, the theory about more recent speciation due to glacial movements seems as likely as any explanation for the variation that is observed in *Reticulitermes* spp. of the Mediterranean region and provides support for Turkish *R. lucifugus* being a different species than European *R. lucifugus.*

The relationship of *R. flavipes* with *R. santonensis* is not surprising. It has long been believed that *R. flavipes* was accidentally introduced from the United States into France and subsequent locations in Europe, including locations in Germany (Weidner 1937, 1951Becker 1970; Harris 1962) and Austria (Heisterberg, 1958, 1959; Hrdý 1961). Recently, Clément et al. (2001) found *R. santonensis* to occur in a sympatric zone with *R. grassei;* and based on its limited distribution (southwestern France), it would appear to be geographically isolated there. Also, on the basis of DNA sequencing analysis of two mtDNA genes and rDNA ITS2, Jenkins et al. (2001) found *R. santonensis* from France to form a close genetic relationship with *R. flavipes* from the United States. The *R. santonensis* analyzed in our study formed a sister clade with samples of *R. flavipes* from a wide geographic range including Texas, Nebraska, Florida, Canada, Germany, and the Bahamas. Our larger geographic range of *R. flavipes*, paired with its similar genetic sequence data obtained in this research lends further support to the aforementioned suppositions that *R. santonensis* may be the result of some limited hybridization event in France from introduced *R. flavipes.*

According to maximum-parsimony and maximum-likelihood analysis, *R. virginicus* and two unidentified groups (Columbia, MO, and Bryan, TX) formed a sister clade to *R. hageni* (Barnesville and Cumberland Island, GA). This relationship between these two species was also observed by Jenkins et al. (2000). Using cuticular hydrocarbon and DNA sequencing of 400 bp of the mtDNA A+T rich region, they found *R. virginicus* to form a sister clade to *R. hageni*, relative to *R. flavipes.* With the maximum-likelihood analysis, *R. hesperus*, *R. hageni*, *R. virginicus*, *R. santonensis*, and *R. flavipes* all formed a common clade (Fig. 2). The common relationship between *Reticulitermes* n. sp. (collected by R. Scheffrahn from Catalina Island, CA), and *R. tibialis* is of interest. Genetic divergence between these two taxa and the inclusion of additional specimens from within its native range may provide more insight into the classification of these taxa.

Although these results suggest that genetic differentiation exists among many *Reticulitermes* spp., by no means do we anticipate that this research will alleviate all of the confusion that has led to the various descriptions of *Reticulitermes*, in particular, the various subspecies of the *lucifugus* complex. This study should assist with a greater knowledge of *Reticulitermes* as a whole, and our results should serve as a baseline for further studies in *Reticulitermes* systematics. Also, the ability of this marker to identify unclassified *Reticulitermes* to species, has potential for the development of PCR-restriction fragment-length polymorphism (RFLP) diagnostics for economically important *Reticulitermes.*

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Analyse comparative et combinée des Reticulitermes *européens*

La systématique des *Reticulitermes* a fait l'objet de plusieurs travaux qui ont remis en cause la classification adoptée par Rossi en 1792 définissant à l'origine une seule espèce de termite *Reticulitermes* (voir la partie "Le genre *Reticulitermes* en Europe : une taxonomie en évolution").

Cette partie de la thèse concerne notamment les travaux axés sur les *Reticulitermes* européens. Grâce à l'utilisation de différentes techniques d'analyse, nous avons réalisé une analyse comparative des différents taxa. Bien que les espèces soient strictement apparentées entre elles, des différences importantes peuvent en effet être observées.

Ces résultats contribuent à éclaircir la taxonomie de ce genre tout en apportant des informations complémentaires sur les mécanismes d'évolution des espèces.

La validité de chacune des techniques comme clef de détermination au niveau spécifique est également discutée. Cet aspect a un grand intérêt si on considère qu'une détermination précise de l'espèce diagnostiquée est nécessaire pour l'application d'une lutte efficace et ciblée. En effet, à large échelle, les *Reticulitermes* ont un impact économique important en milieu urbain, qui a été estimé à un milliard d'Euros en 2005 en Europe selon le rapport UNEP-FAO.

Dans **l'article III**, nous présentons des données récentes analysées avec celles obtenues précédemment par notre équipe. Cette étude concerne donc le comportement d'agression interspécifique, la composition des hydrocarbures cuticulaires, la détermination des substances défensives de la glande frontale des soldats et le séquençage du fragment d'ADN mitochondrial ND1.

Le comportement d'agression contribue à l'isolement spécifique puisqu'il empêche la fusion de colonies et la conséquente hybridation des reproducteurs secondaires néoteniques. Cet isolement spécifique est confirmé par les autres résultats qui révèlent la présence de six phénotypes en Europe, correspondant aux différentes espèces. Les populations corses, proches de l'espèce italienne, ont le statut de sous-espèce en raison de leur isolement géographique.

L'article IV, réalisé en collaboration avec Alexandre Quintana (étudiant en thèse, financement CNRS/Laboratoire Pierre Fabre), concerne l'analyse des terpènes à partir des sécrétions de la glande frontale des soldats. Cette sécrétion est expulsée en cas de défense de la colonie à travers un pore situé sur la partie antérieure de la tête.

En Europe, cette sécrétion a fait l'objet de plusieurs travaux (voir par exemple Parton et al., 1981 ; Bagnères et al., 1990), mais seuls les composés les plus abondants avaient été analysés, les autres présents en quantité inférieure étant à l'époque difficiles à déterminer. La composition de la sécrétion varie en fonction de l'espèce et de la localisation géographique, et a déjà été employée pour des révisions systématiques des *Reticulitermes* nord-américains (Haverty, et al. 1996, 1999 ; Nelson et al., 2001).

Les profils chimiques obtenus permettent une séparation univoque des espèces. Une analyse comparée des profils confirme également une origine monophylétique du nouveau phénotype *R. sp. nov.* et l'espèce présente dans les Balkans, *R. balkanensis*.

Le **dernier article** (**V**) de ce chapitre, en préparation, présente un intérêt particulier pour son approche méthodologique. En effet, les relations phylogénétiques au sein des *Reticulitermes* ont été étudiées à l'aide d'une analyse combinée des deux caractères hydrocarbures cuticulaires / séquences nucléotidiques. Ces caractères ont déjà été étudiés avec une approche comparative (Jenkins et al., 2000), mais une analyse combinée n'avait pas encore été réalisée. L'approche que nous proposons dans cette publication est basée sur une utilisation conjointe des données qui peut augmenter le support des arbres phylogénétiques obtenus à des différents niveaux (approche de *Total Evidence*, Kluge, 1989). Les avantages de cette méthode vis à vis d'une analyse séparée hydrocarbures cuticulaires / séquences d'ADN y sont également discutés.

- **Article III** : Clément, J.-L., A.-G. Bagnères, **P. Uva**, L. Wilfert, A. Quintana, J. Reinhard and S. Dronnet, 2001. Biosystematics of *Reticulitermes* termites in Europe: morphological, chemical and molecular data. *Insectes Sociaux* 48 : 202-215.

- **Artilcle IV** : Quintana, A., J. Reinhard, R. Faure, **P. Uva**, A.-G. Bagnères, G. Massiot and J.-L. Clément. Geographic variation in terpenoid composition of European *Reticulitermes* termite defensive secretions. Soumis à *Journal of Chemical Ecology.*
- **Article V : Uva, P.** and A.-G. Bagnères. Phylogeny of European *Reticulitermes*: a total evidence approach. En préparation.

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Insectes Sociaux

Research article

Biosystematics of *Reticulitermes* termites in Europe: morphological, chemical and molecular data

Dedicated to Professor Ch. Noirot

J.-L. Clément[3], A.-G. Bagnères[3], P. Uva[3], L. Wilfert[2], A. Quintana[1], J. Reinhard[1] and S. Dronnet[1]

[1] *Laboratoire de Neurobiologie, Centre National de la Recherche Scientifique, UPR 9024, Communication chimique, 31 chemin J. Aiguier, 13402 Marseille, France, e-mail: comchim@lnb.cnrs-mrs.fr*
[2] *Present address: Department of Animal Physiology, University of Bayreuth, 95440, Bayreuth, Germany*
[3] *Present address: Institut de Recherche sur la Biologie de l'Insecte, Centre National de la Recherche Scientifique Université F. Rabelais, UMR 6035, Parc Grandmont, 37200 Tours, France*

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Summary. In Europe the most abundant naturally residing termite is the subterranean genus *Reticulitermes* (Rhinotermitidae). Six phenotypes of *Reticulitermes* have been identified on the basis of morphological, chemical (cuticular hydrocarbons and soldier defensive secretions), and molecular (enzymatic alleles and mitochondrial ND1 sequence) features. They are *R. santonensis* in western France, *R. grassei* in southwestern France, northwestern and southern Spain and Portugal, *R. banyulensis* in northeastern Spain, central area of the Iberian Peninsula and southwestern France, *R. lucifugus* in Italy and southeastern France, *R. balkanensis* in the Balkans and *R. sp. nov.,* a recently identified urban phenotype resembling *R. balkanensis*, in northern Italy and southeastern France. *R. santonensis* is close kin to the American species *R. flavipes. R. grassei*, *R. banyulensis and R. lucifugus* belong to the same species complex. *R. balkanensis* and the new phenotype *R. sp. nov.* are close to *R. santonensis* regarding cuticular hydrocarbons, to the *lucifugus* complex regarding DNA and to *R. clypeatus* from Israel regarding morphology. The species status of these genotypes has been confirmed by the mechanisms of species isolation. Prevention of hybridization depends on the method of colony formation in each species. Swarming dates, differences in pheromones, and infertility prevent hybridization by sexual alates. Interspecific aggression between workers prevents hybridization by neotenics. Behavioral and molecular studies have provided many data on the genetic structure of nests, which varies according to species and location. All colonies of *R. santonensis* are open all year. The colonies of *R. grassei* in southern areas and all colonies of *R. banyulensis* are closed families with generally a single reproductive couple. The colonies of *R. grassei* in northern areas and the colonies of *R. lucifugus* are open in the summer and closed in the winter. Based on the here presented data, the taxonomy and the speciation of the *Reticulitermes* genus in Europe are discussed.

Key words: Termites, *Reticulitermes*, taxonomy, Europe, species isolation.

Introduction

Termite taxonomy is a complex issue and has been subject matter to long controversies. For many years *Reticulitermes lucifugus* (Rhinotermitidae) described by Rossi (1792) was believed to be the only termite species residing naturally in Europe other than *Kalotermes flavicollis* (Kalotermitidae). In 1837 Kollar identified a second *Reticulitermes* species, i. e. *R. flavipes,* in greenhouse specimens collected in a greenhouse at the Schönbrunn Palace, Vienna, Austria. Although some authors subsequently speculated that *R. flavipes* was imported from the United States, this hypothesis cannot be validated because the original description is incomplete. Boffinet (1842) and Quatrefages (1853) described large urban populations of *Reticulitermes lucifugus* in Rochefort, La Rochelle and many towns in Charente (France). Based on major behavioral differences between *R. lucifugus* populations in France, Feytaud (1924) and Grassé (1954) concluded that populations living in southern France and Charente constituted two different races. In 1924, Feytaud described the Charente populations as a subspecies of *R. lucifugus* named *R. l. santonensis.* Feytaud (1925) and Jucci (1924) compared this subspecies to the American species *R. flavipes* and concluded that they were very similar, perhaps the same species. In his 1952 report including a description of the species *R. clypeatus* based on a specimen brought back

from Jerusalem, Lash compared the different *Reticulitermes* termites in his possession and concluded that *R. flavipes* and *R. santonensis* were not the same. Jacquiot (1955) described urban populations of *R. santonensis* in Paris and many towns in France. In 1963, Cals-Usciati Frescheville described *R. lucifugus* populations in Paris. Many authors described *R. flavipes* populations in Germany (Weidner 1937, 1951; Becker, 1970; Harris, 1962) and in Austria (Heisterberg, 1958, 1959; Hrdy, 1961). In detailed studies using powerful biochemical, biometric, chemical, and genetic techniques, Clément and coworkers distinguished criteria that elevated *R. santonensis* to the rank of species and identified sibling species in the *lucifugus* complex (Clément, 1977a, b, 1978b, 1979a, 1981b, c, 1982a, b, c, e, 1984; Clément et al., 1986). Furthermore, comparison of *R. santonensis* and *R. flavipes* based on chemical analyses of cuticular hydrocarbons and soldier defense compounds (Bagnères et al., 1990a) showed the existence of numerous phenotypes in *R. flavipes*, none identical to *R. santonensis*.

Morphological differences are great between genera but not between species (Banks and Snyder, 1920; Krishna, 1970). As a result, accurate identification of phenotypes was unfeasible until additional scientific technology allowed evaluation of biochemical and molecular features (Clément, 1981c; Parton et al., 1981; Clément et al., 1986; Howard et al., 1982; Lange et al., 1989; Crozier, 1990; Luykx, 1993; Brughton, 1995; Haverty and Nelson, 1997; Haverty et al., 1991, 1996, 1999; Kambhampati et al., 1996; Husseneder et al., 1998; Jenkins et al., 1998, 1999, 2000; Reilly, 1987; Thompson and Hebert, 1998; Vargo, 2000). A total of six *Reticulitermes* phenotypes were subsequently identified in Europe and many others in the United States. In accordance with the biological definition of species proposed by Mayr (1963), the species status of these phenotypes has been confirmed by ascertaining species isolation.

The purpose of this study is to review available data on European *Reticulitermes* phenotypes and describe new data on the mechanisms of isolation (Bouillon, 1981). The discussion deals with distribution areas in natural and urban zones, with genetic structure of the different populations and species, and with the different hypotheses proposed to explain evolution and speciation.

Materials and methods

Specimen collection

Two series of specimens were used in this study. The first was collected in France, Spain, Portugal, Italy, and Greece between 1976 and 1985 to study morphology (more than 1000 sexual alates), enzymatic polymorphism (104 colonies) except *R. sp.*, initial studies of the frontal glands (380 soldiers) and cuticular hydrocarbons except *R. balkanensis* and *R. sp.* The other series was used to obtain data concerning morphology, cuticular hydrocarbons, frontal gland defensive compounds, DNA sequencing, and mathematical analysis of cuticular hydrocarbons between 1986 and 2000. This second series of specimens included natural and urban sites:

– *R. santonensis* (31 colonies) from France, except samples from Chile and Germany;
– *R. grassei* (53 colonies) from France and Spain;
– *R. banyulensis* (49 colonies) from France and Spain;
– *R. lucifugus* (34 colonies) from France and Italy (including Sicily);
– *R. lucifugus corsicus* (7 colonies) from various sites of Corsica and Sardinia;
– *R. balkanensis* (7 colonies) from Greece;
– *R. sp. nov.* (10 colonies) mainly from urban locations in southeastern France and Italy since 1998;
– *R. flavipes* (26 colonies) from natural environment in USA (Georgia, Mississippi, and North Carolina) between 1983 to 2000.

Specimens of each of the *Reticulitermes* species described in this paper will be sent to the Museum d'Histoire Naturelle in Paris, France as voucher specimens.

Morphology

Scanning electron microscopic pictures (coronal and profile views) were made of the heads for each species (Clément, 1978b; Bagnères et al., 1990a) and studied using a binocular microscope to evaluate the profile of the postclypeus. Color of the tibia of reproductives was also noted because this feature is an informative morphological indicator in association with the postclypeus profile.

Chemistry

GLC and GC-MS analyses. Analyses were carried out on *R. santonensis* (Parton et al., 1981; Lemaire and Clément, 1987; Bagnères et al., 1988, 1990a), on *R. flavipes* (Bagnères et al., 1990a) and on *R. grassei* and *R. banyulensis* (Parton et al., 1981; Lemaire et al., 1987; Bagnères et al., 1988, 1990a, 1991). *R. lucifugus* had been studied by Uva and Bagnères (Uva, 1999). For the present study, GLC analyses using single or groups of ten individuals and GC-MS analyses using pooled individuals were repeated on all species. GLC was performed on a Delsi Nermag DN 200 GLC coupled with an Enica 31 integrator using the same temperature programs and column as published by Bagnères et al. (1990a, 1991). GC-MS analysis was performed using the same temperature programs and column on a Hewlett-Packard 5890 GLC system coupled to a 5989A MS, controlled by a HPUX chemstation. The electron impact (70 eV) mode was operated with m/z scanning range from 40 to 600.

Mathematical analyses. Hydrocarbon peaks corresponding to each species were analyzed as described elsewhere (Bagnères et al., 1988, 1990a, 1991). Relative proportions were determined using an Excel spreadsheet macro and then transferred into a Statgraphics matrix (Statgraphics v. 4.0 and Uniwin Plus v. 3.0) to perform various principal component analyses (PCA) and hierarchical ascendant classification (HAC). Two PCAs were carried out: one using all individuals (187) of each species and the other using only individuals with one hydrocarbon phenotype, i.e. that of the *santonensis* group including *R. santonensis*, *R. flavipes*, *R. balkanensis*, and *R. sp.* HAC analyses were carried out with different subgroups obtained on the basis of barycenters in PCA.

Molecular genetics

Enzymatic polymorphism methods were described by Clément (1981a, 1984). DNA extraction was performed using a modified version of the method described by Kocher et al. (1989). Amplification of mitochondrial DNA (mtDNA) was achieved by polymerase chain reaction (PCR) using primers in the 16s rRNA gene (5′-CGTTTCGATCATTAAAATCTTAC-3′) and NADH dehydrogenase 1 (ND1) gene (5′-ATCAAAAGGAGCTCGATTAGTTTC-3′). This region (742 bp) harbors part of the ND1 gene, the tRNA Leu gene, and part of the 16 s rRNA gene. The cycling program included an initial two-minute denaturation cycle at 92 °C, followed by 40 cycles of 15 s at 92 °C, 45 s at 50 °C, 2 min at

62 °C, and finally a 7-minute cycle at 62 °C. Multiple consensus sequences were aligned with BIOEDIT 4.8.10 (Hall, 1999). Phylogenetic analysis was performed using the DNADIST (Kimura 2-parameter model) and NEIGHBOR applications in the PHYLIPS 3.5c package (Felsenstein, 1993) to obtain a neighbor-joining tree (Saitou and Nei, 1987). Trees were drawn with TREEVIEW (Page, 1996).

Behavioral tests

Long-distance attraction and contact sex-pheromone tests were carried out in an olfactometer (Clément, 1982a). A total of 600 male and female *R. santonensis*, *R. grassei,* and *R. banyulensis* were tested. Results of aggression tests were taken from various sources (Clément, 1980, 1987a; Clément and Bagnères, 1998; Bagnères, 1989; Uva, 1999). Recent tests (1999–2000) were performed in the winter between October and March and summer between April and September. Behavioral tests were performed at the latest two months after field collection. As far as possible, the goal was to obtain complete test results for both seasons.

Results

The techniques used in this study identified six different termite phenotypes. Assessment of the mechanisms of reproductive isolation showed that these phenotypes could be considered separate species. For sake of clarity, species and phenotype names are used synonymously throughout this presentation. The six phenotypes identified were as follows:

- *Reticulitermes santonensis* Feytaud (**S**), in France (Charente-maritime, Landes, and urban locations);
- *Reticulitermes grassei* Clément (**G**), in France, Spain, and Portugal. *R. grassei* is sympatric with *R. santonensis* in France and *R. banyulensis* in Spain;
- *Reticulitermes banyulensis* Clément (**B**), in Spain and southwestern France. *R. banyulensis* is sympatric with *R. grassei*, in France and Spain;
- *Reticulitermes lucifugus* Rossi (**L**), in France (Provence) and Italy. Our results showed that natural populations in Corsica (*R. l. corsicus*) (**C**) have a phenotype very close to that of *R. lucifugus*;
- *Reticulitermes balkanensis* Clément (**Bk**), in Greece and Albania;
- In addition a phenotype displaying a phenotype similar to *R. balkanensis* (morphology and cuticular hydrocarbons) but with slight differences in soldier defensive compounds was identified in some urban zones in Italy and France (Alpes-Maritimes, Bouches-du-Rhône, Isère and Var). In the following text this phenotype will be referred to as *R. sp. nov.* (**Sp**).

Taxonomic criteria

Morphology. *R. santonensis* is distinguishable from the other species by the shape of its postclypeus, which is straight in both workers and reproductives (Clément, 1978b). *Santonensis* reproductives have yellow tibia as described by Feytaud (1924) and Clément (1978b). This phenotype is the same as *R. flavipes*. In *R. grassei*, *R. banyulensis*, *R. lucifugus*, and *R. balkanensis*, workers and reproductives present a markedly curved postclypeus. On lateral views the postclypeus of *R. grassei* and *R. lucifugus* is flat while that of *R. banyulensis* and *R. balkanensis* is more prominent. In *R. balkanensis* and *R. sp.,* the top of the postclypeus is higher than the top of the head (Fig. 1). In *R. sp.* the postclypeus is similar to that described by Lash (1952) for *R. clypeatus* from Jerusalem. The tibia of reproductives in the *lucifugus* group are black or brown, except in the *corsicus* subgroup which is characterized by a yellow tibia (Table 1).

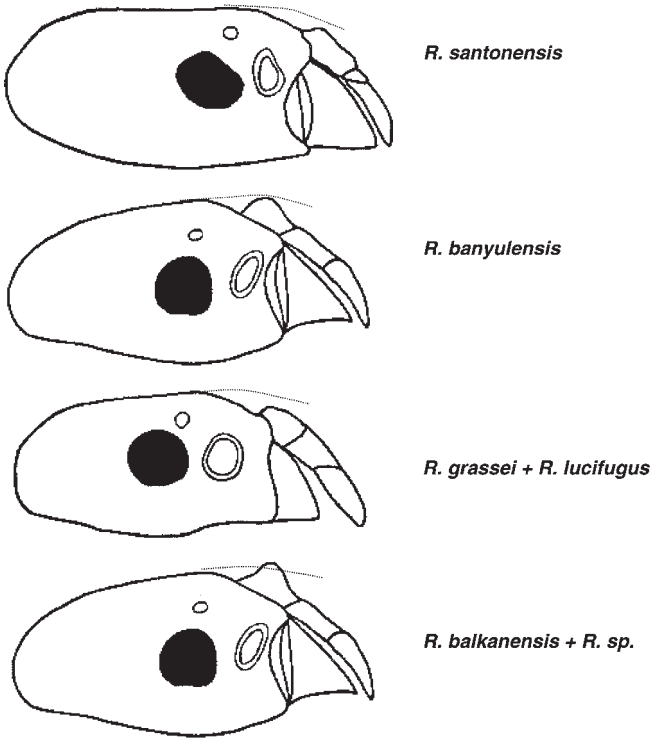

Figure 1. Lateral aspect of the heads of sexual alates showing the four different positions of the postclypeus

Defensive compounds from soldier frontal glands. Reticulitermes soldiers have prominent mandibles and a frontal gland (Noirot, 1969). Defensive compounds are produced by the frontal gland and stored in a reservoir also located in the head. These secretions are unaffected by diet (filter paper, various wood essences). The amount secreted by the soldiers (Parton et al., 1981) is correlated with predators in that ecosystem (Lemaire and Clément, 1987; Lemaire et al., 1990).

As in other termite genera (Prestwich, 1979, 1984), the extracts from defensive glands of *Reticulitermes* soldiers are different between species. The compounds are monoterpenes (α-pinene, β-pinene, limonene), sesquiterpenes, not yet characterized, and one diterpene alcohol, i.e. geranyl linalool (Parton et al., 1981; Baker et al., 1982). The results of chemical analysis of soldier defensive compounds for each phenotype are shown in Table 2 and Figure 2.

R. santonensis and the "a", "c", and "e" phenotypes of *R. flavipes* (F) display large quantities of monoterpenes

Table 1. Morphology of sexual alates

Species	Post-clypeus					Tibia	
	Top view		Side view			Yellow	Dark
	Flat	Curved	Flat	Intermediate	Curved		
R. santonensis	+		+			+	
R. flavipes	+		+			+	
R. sp.		+			+		+
R. balkanensis		+			+		+
R. lucifugus		+	+				+
R. l. corsicus		+	+			+	
R. banyulensis		+		+			+
R. grassei		+	+				+

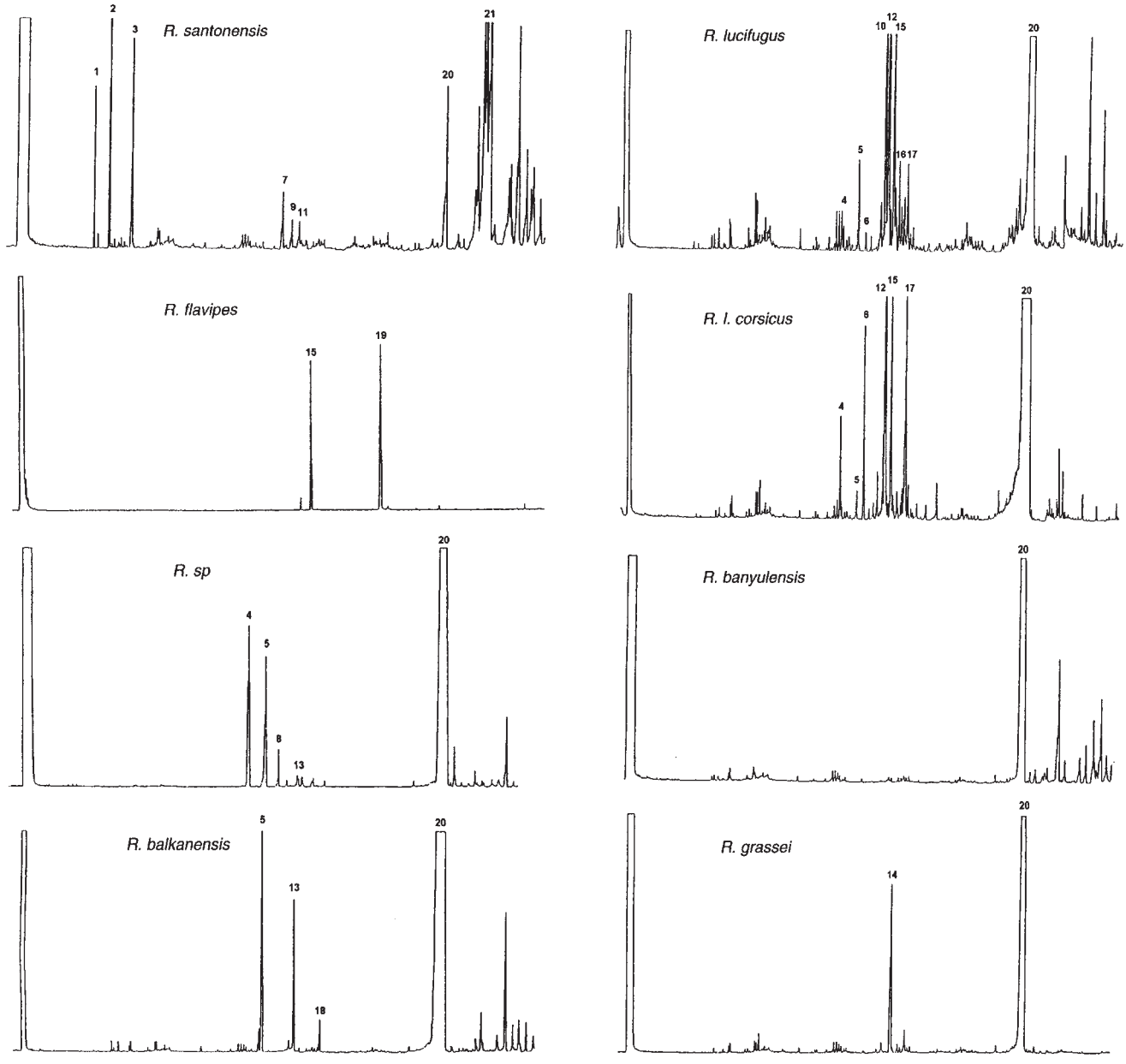

Figure 2. Gas chromatograms of defensive compounds extracted using pentane from soldiers with different phenotypes. Peak numbers are explained in Table 2

(Parton et al., 1981; Clément et al., 1988; Bagnères et al., 1990a). The other species have no monoterpenes.

R. banyulensis has no sesquiterpenes. *R. grassei* displays one sesquiterpene in large quantity. The sesquiterpenes are present in large quantities in all other species but with numerous qualitative differences except with regard to peak n° 5 in *R. sp., R. balkanensis, R. lucifugus* and *R. l. corsicus*. The sesquiterpenes observed in *R. santonensis* are different from those exhibited in the *R. flavipes* phenotypes. The *flavipes* phenotype shown here corresponds to the "b" phenotype described by Bagnères et al. (1990a) and also to *R. flavipes* described by Zalkov et al. (1981) with γ cadinene (peak n° 15) and its aldehyde (peak n° 19). The sesquiterpene mixture in *R. sp.* is comparatively distinct from the one of *R. balkanensis*. The differences between *R. lucifugus* and *R. lucifugus corsicus* are strictly quantitative. These findings indicate sesquiterpene analysis is an excellent diagnostic tool.

Although reportedly absent in some American phenotypes of *R. flavipes* (Bagnères et al., 1990a), geranyl linalool was found in variable amounts in all European species studied. This compound is present in low quantity in *R. santonensis* in association with at least one other more abundant unidentified diterpene alcohol (peak n° 21). Further studies (NMR, GC/MS/MS) are now in progress to ascertain the exact chemical structure of these molecules.

Cuticular hydrocarbons. When nestmates meet in a tunnel, they perform a sequence of basic recognition movements while they examine cuticular hydrocarbons (Clément, 1981a, 1982d, 1984; Clément and Bagnères, 1998). This system has been successfully simulated using an artificial neuronal network to identify different *Reticulitermes* species and castes based on variations in the relative proportion of cuticular hydrocarbons (Bagnères et al., 1988, 1998).

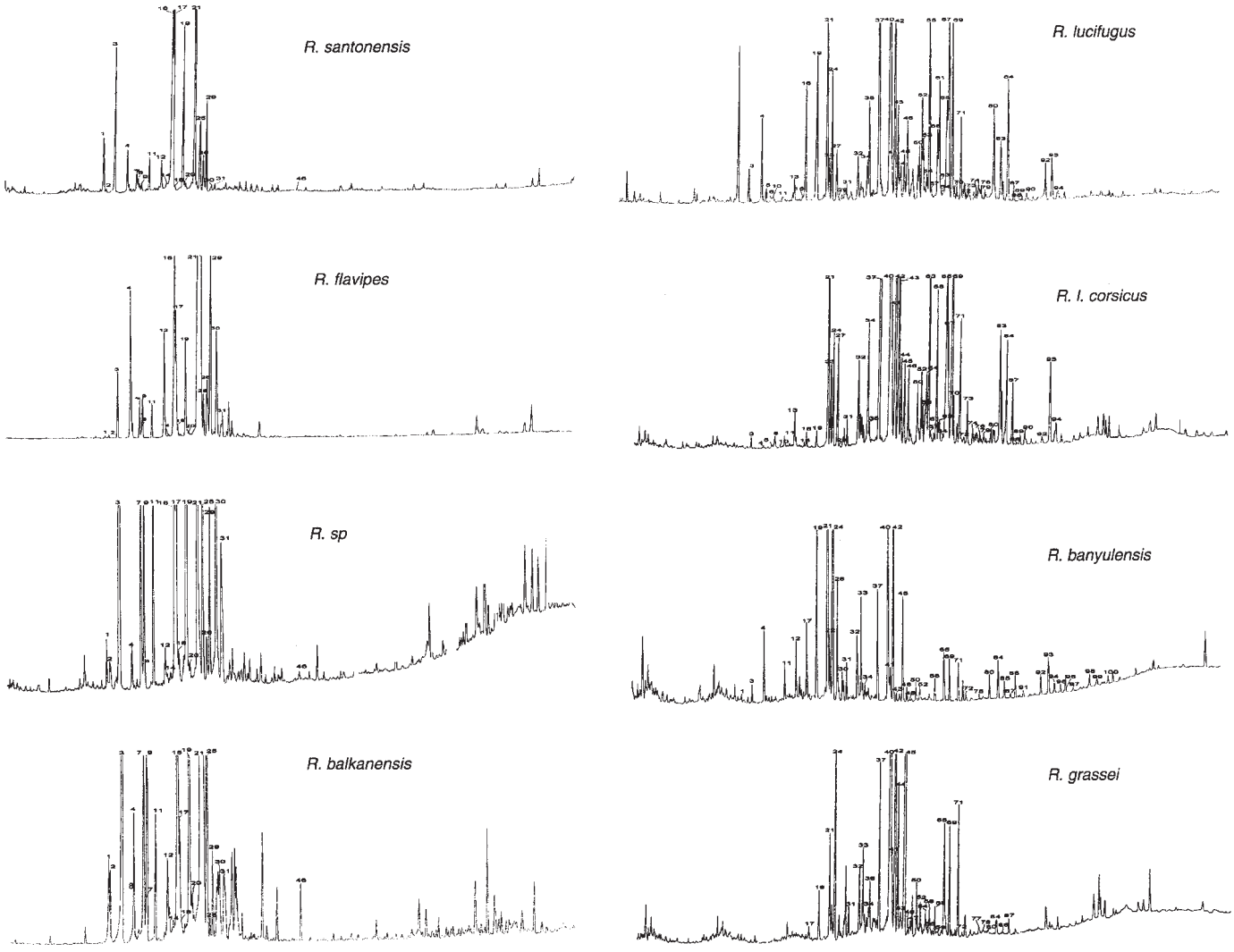

Figure 3. Gas chromatograms of cuticular hydrocarbons extracted using pentane from workers with different phenotypes. Peak numbers are explained in Table 3

Table 2. Defensive compounds identified in European *Reticulitermes* termites soldiers. The compounds identified are **1**: *α*-pinene, **2**: *β*-pinene, **3**: limonene, **15**: *γ* cadinene, **19**: cadinene aldehyde, **20**: geranyl-linalool. Abbreviations: M: monoterpene, S: sesquiterpene, D: diterpene, and **Rt**: retention time in minutes, **S**: *R. santonensis*, **Fb**: "b" phenotype of *R. flavipes,* **Sp**: *R. sp.,* **Bk**: *R. balkanensis,* **L**: *R. lucifugus* **C**: *R. lucifugus corsicus*, **B**: *R. banyulensis*, **G**: *R. grassei*, Symbols: +: minor component, ++: abundant component, +++: major component

N°	Terpenoids	Rt	S	Fb	Sp	Bk	L	C	B	G
1	M	7.41	+++							
2	M	8.68	+++							
3	M	10.47	+++							
4	S	20.3			++		+	++		
5	S	21.7			++	+++	++	+		
6	S	21.82					+	+++		
7	S	22.37	+							
8	S	22.5			+					
9	S	23.09	+							
10	S	23.47					+++			
11	S	23.68	+							
12	S	23.74					+++	+++		
13	S	23.93			+	++				
14	S	24.1								+++
15	S	24.17		+++			+++	+++		
16	S	24.62					++			
17	S	25.38					++	+++		
18	S	26.04				+				
19	S	29.31		+++						
20	D	35.02	++		+++	+++	+++	+++	+++	+++
21	D	38.28	+++							

Just as termites presumably do, we can use cuticular hydrocarbons as a tool for species identification. Cuticular blends can contain between 30 and 100 different hydrocarbons including linear, monomethyl, or dimethyl alkanes or alkenes (Lange et al., 1989; Bagnères et al., 1990a, 1991). The relative proportions of these molecules are controlled but the exact nature of the regulation process is unclear (Bagnères et al., 1996; Vauchot et al., 1996, 1997, 1998; Sevala et al., 2000). Qualitative differences in cuticular blends (Fig. 3) are used to discriminate *Reticulitermes* phenotypes in Europe and the United States (Howard et al., 1978, 1982; Bagnères et al., 1990b, 1991b; Haverty and Nelson, 1997; Haverty et al., 1991, 1996, 1999). The molecules found on the cuticle of *Reticulitermes* termites are listed in Table 3. Cuticular analyses of the termite population in Kent (UK) showed that its chemical signature corresponded to *R. grassei*.

Multivariate principal components analyses (PCA) using relative proportions of cuticular hydrocarbons are used to characterize phenotypes. PCA was performed using the hydrocarbons from all phenotypes (Fig. 4). The first two canonical axes of the projection accounted for 48% of the variation and discriminated four groups of termites. The first group included *R. santonensis*, *R. flavipes*, *R. balkanensis*, and *R. sp.* phenotypes; the second the *R. banyulensis* phenotype; the third the *R. grassei* phenotype; and the fourth the *R. lucifugus* and the *corsicus* subspecies phenotypes.

When PCA was performed using hydrocarbons from individuals displaying *R. santonensis*, *R. flavipes*, *R. balkanensis*, and *R. sp*. phenotype, the first two canonical axes accounted for 45% of variation. *R. sp.* appeared on the positive side of the axes, *R. santonensis* and the "a" and "b" phenotypes of *R. flavipes* appeared in the center (Fig. 5). The other phenotypes of *R. flavipes* were projected on the negative side of axis 1. These findings are in agreement with those published by Bagnères et al. (1990a). *R. sp.* was projected with *R. balkanensis*. To determine to which group the termites from Hamburg (Germany) and Santiago (Chili) belonged, we projected these as free variables, i.e., after creation of the new canonical axes. Under these conditions, they appeared in the space defined by *R. santonensis* as indicated by the arrows on the PCA in Fig. 5. This phenotype similarity was confirmed by comparison of defensive compound extracts.

Enzymatic data. Diagnostic alleles were discovered by electrophoresis of esterase and phosphatase acids enzymatic proteins. Each species is characterized by alleles that distinguish sympatric or allopatric sibling species (Clément, 1981c, 1984). Differences in allele frequency can be used to discriminate between populations and to show if hybridization takes place in sympatric zones. Calculation of genetic distances (Nei, 1972) has established kinship between *R. banyulensis* and *R. lucifugus*. *R. balkanensis* is also close to this group. *R. grassei* is close to the group formed by *R. banyulensis*, *R. lucifugus*, and *R. balkanensis.*

Mitochondrial DNA data. Analysis of ND1 mtDNA was in agreement with morphological and chemical studies and allowed construction of a neighbor joining tree that can be used to suggest similarities between species (Fig. 6). Using the method proposed by Kambhampati et al. (1996) with *Coptotermes formosanus* as the outgroup, four clades are produced. *R. santonensis* forms a clade with *R. flavipes* from north Carolina (phenotype b), *R. grassei* with *R. banyulensis*, *R. balkanensis* with *R. sp.* and *R. lucifugus corsicus* with *R. lucifugus*.

Mechanisms of species isolation

Species isolation mechanisms of alate reproduction involve the date of swarming, long distance sex pheromones, contact pheromones used during tandem formation, and infertility of the partners or their descendants. Clément (1979b) showed that viable hybrids could be obtained between sympatric *R. grassei* and *R. santonensis* if the date of swarming was artificially modified although these conditions are unlikely to occur under natural conditions. For neotenic reproduction, species isolation depends on interspecific aggression between workers (Clément 1978a, 1980, 1982b, d; Clément and Lange, 1984; Clément et al., 1986; Thorne, 1982; Thorne and Haverty, 1991). The possibility of hybridization by neotenics can be investigated by aggression tests using different phenotypes.

Table 3. Cuticular hydrocarbons from *Reticulitermes* workers. Abbreviations: **ECL**: equivalent chain length, **S**: *R. santonensis,* **F**: *R. flavipes,* **Bk:** *R. balkanensis*, **Sp**: *R. sp*., **B**: *R. banyulensis,* **G**: *R. grassei*, **L**: *R. lucifugus,* **C**: *R. lucifugus corsicus*

	Compound	ECL	S/F/Bk/Sp	B	G	L/C
1	9-C23:1	22.66	×	×		
2	x-C23:1	22.74	×			
3	n-C23	23.00	×	×		×
4	11-MeC23	23.36	×	×		×
5	5-MeC23	23.53				×
6	Unknown	23.59				×
7	4/2-MeC23	23.63	×			
8	z,9-C24:1	23.69	×			
9	3-MeC23	23.72	×			
10	x,y-diMeC23	23.73				×
11	n-C24	24.00	×	×		×
12	11/12-MeC24	24.35	×	×		
13	Unknown	24.37				×
14	5-MeC24	24.53	×			
15	x-C25:1	24.64				×
16	4/2-MeC24	24.65	×			×
17	z,9-C25:1	24.71	×	×	×	
18	x-C25:1	24.79	×			
19	*n*-C25	25.00	×	×	×	×
20	x15	25.06	×			
21	11/13-MeC25	*25.35*	×	×	×	×
22	9-MeC25	25.44		×		
23	Unknown	25.44				×
24	5-MeC25	25.53		×	×	×
25	7,9-C25:2	25.55	×			
26	4/2-MeC25	25.65	×			
27	11,15-diMeC25	25.66				×
28	9,13-diMeC25	25.69		×		
29	3-MeC25	25.75	×			×
30	5,17-diMeC25	25.91	×	×		
31	n-C26	26.00	×	×	×	×
32	13/12/11-MeC26+ x-C27:1	26.35		×	×	×
33	6-MeC26	26.47		×	×	
34	4/2-C26	26.64		×	×	×
35	x-C27:1	26.70				×
36	9-C27:1	26.71			×	
37	n-C27	27.00		×	×	×
38	x,y-C27:2	27.08				
39	z,w-C27:2	27.13				
40	13/11-MeC27	27.33		×	×	×
41	7-MeC27 (+ x-C27:2)	27.43		×	×	×
42	5-MeC27	27.52		×	×	×
43	3-MeC27	27.63		×	×	×
44	11,15-diMeC27 + 4/2 MeC27	27.63			×	×
45	5,17-diMeC27	27.87		×	×	×
46	*n*-C28 + *unknown*	*28.00*	×	×	×	×
47	Unknown	28.01			×	
48	Unknown	28.09		×		
49	x,y-C29:2	28.30				
50	15/13/11-MeC28	28.34		×	×	×
51	z,w-C29:2	28.38				
52	6-MeC28	28.46		×	×	×
53	5-MeC28	28.52				×
54	4/2-MeC28	28.65			×	×
55	9-C29:1	28.72			×	×
56	3-MeC28	28.79			×	
57	x-C29:3	28.80				×
58	*n*-C29	29.00		×	×	×
59	Unknown	29.01			×	
60	x,y,z-C29:3	29.03				
61	x-C29:3	29.06				×
62	u,v,w-C29:3	29.12				
63	x-C29:2	29.13				×
64	x-C29:2	29.26				×
65	15/13/11-MeC29	29.32		×	×	×
66	x,y-C30:2	29.32				
67	x-C29:2	29.39				×
68	u,v-C29:2	29.41				
69	5/7-MeC29	29.52		×	×	×
70	x-C29:2	29.60				×
71	5,17-diMeC27	29.82		×	×	×
72	*n*-C30	30.00		×	×	×
73	x,y-diMeC29	30.11				×
74	15/14/13/12/11-MeC30	30.32				×
75	5-MeC30	30.57		×		
76	14,18/12,16-diMeC30 + 2-MeC30	30.59				×
77	6-MeC30	30.61			×	
78	5,17-diMeC30	30.70			×	
79	x-C31:3	30.76				×
80	n-C31	31.00		×	×	×
81	x,y,z-C31:3	31.01				
82	x-C31:3	31.05				×
83	13,17-diMeC31	31.05				×
84	15/13/11-MeC31	31.31		×	×	×
85	5-MeC31	31.56		×		
86	Unknown	31.56			×	
87	5,17-diMeC31	31.81		×	×	×
88	*n*-C32	32.00		×		×
89	x,y-diMeC31	32.15				×
90	14-MeC32	32.28				×
91	12-MeC32	32.30		×		
92	*n*-C33	33.00		×		×
93	15/13-MeC33	33.30		×		×
94	5-MeC33+ 13,17-diMeC33+ 13,19/11,21-diMeC33	33.50		×		×
95	Unknown	33.70		×		
96	*n*-C34	34.00		×		
97	13/11-MeC34	34.30		×		
98	n-C35	35.00		×		
99	13/11-MeC35	35.32		×		
100	Unknown	35.80		×		

Winged sexual alates. Personal observations show that isolation is absolute between *R. grassei* and *R. banyulensis* that swarm at the same time in the sympatric area (Fig. 7). Clément (1982a) has shown that *R. grassei* and *R. banyulensis* are isolated by long-distance sex pheromones from the sternal gland, with the pheromonal composition of *R. banyulensis* still unclear. Even under experimental conditions involving forced simultaneous swarming and artificial mixing in the same zone, male behavior during tandem formation further strengthens species isolation since most couples are homospecific (Clément, 1982). *R. grassei* and *R. santonensis* are not isolated by long-distance pheromones in sympatric

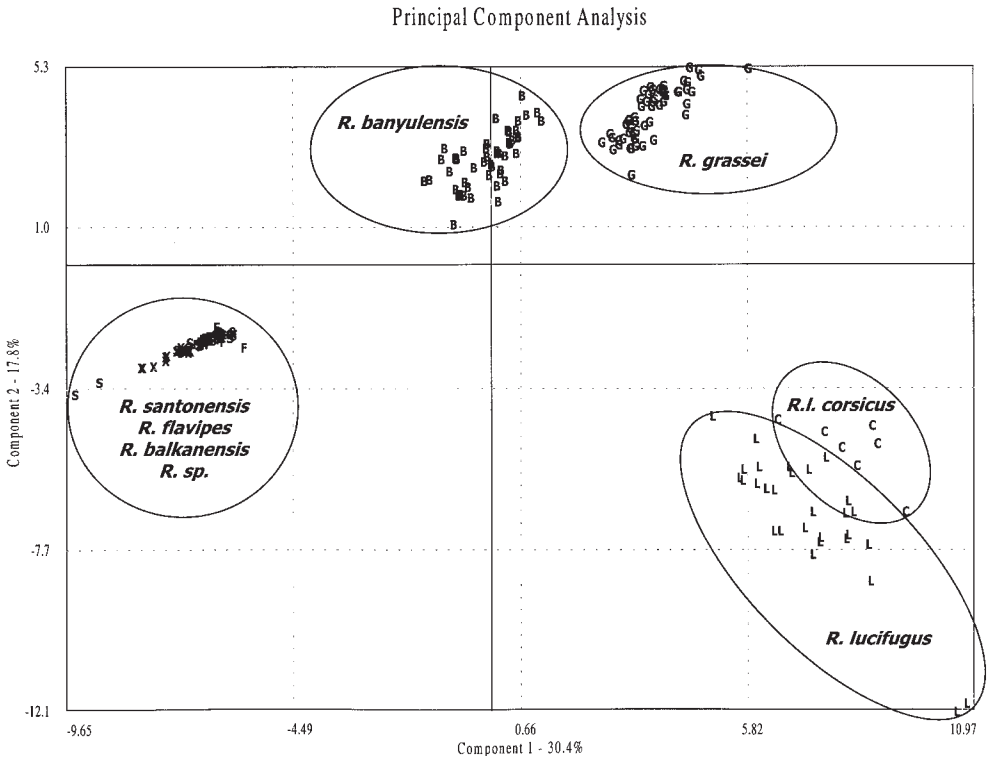

Figure 4. Principal Component Analysis projection using the relative proportion of hydrocarbons from *Reticulitermes* workers on the first two canonical axes. The circles surrounding each group were not calculated according to statistical distribution

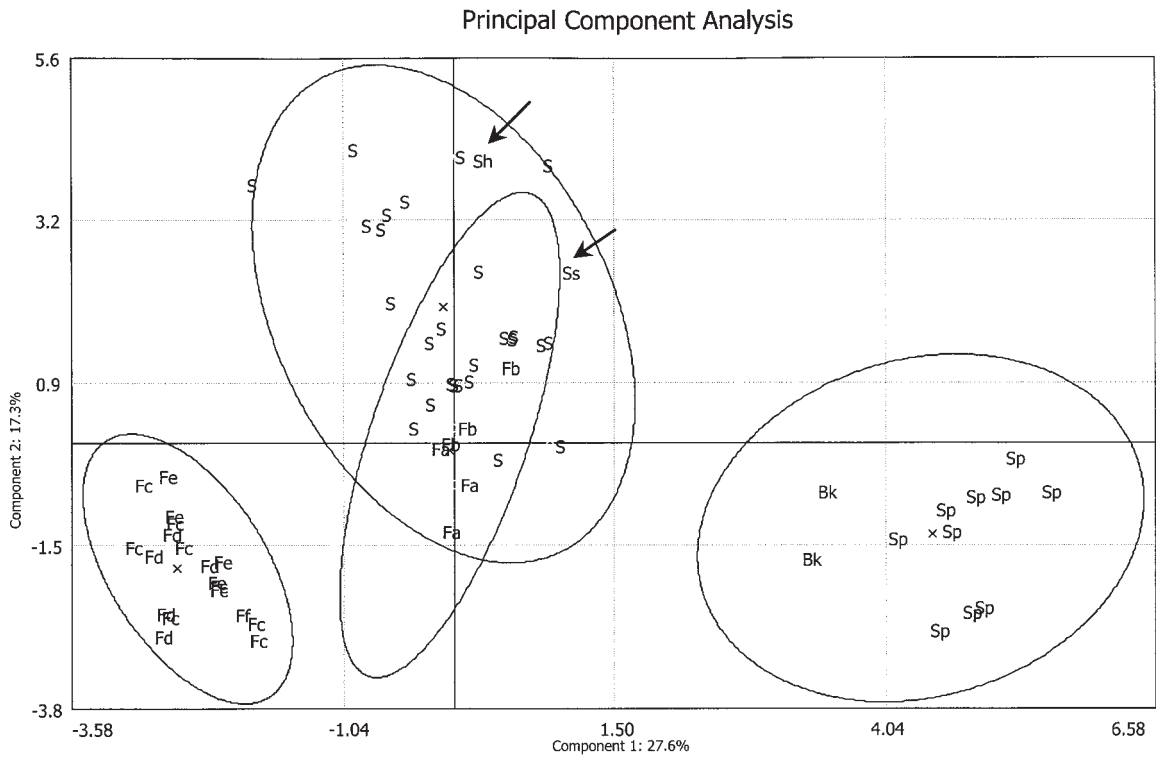

Figure 5. Principal Component Analysis projection using the relative proportion of hydrocarbons from *Reticulitermes* workers in the *santonensis-flavipes-balkanensis* group on the first two canonical axes. **S** = *R. santonensis*; **Fa, Fb, Fc, Fd, Fe,** and **Ff** stand for the different phenotypes of *R. flavipes* found in the southeastern United States; **Bk** = *R. balkanensis*; **Sp** = *R. sp.*; and **Ss** = specimen from Santiago, Chile and **Sh** = Hamburg, Germany. The arrows indicate that the two samples were projected as free variables and not used for the calculation of the new canonical axes. The ellipses are 99% confidence ellipses. The × mark indicates the barycenter of the ellipse

Figure 6. Neighbor-Joining tree using ND1 mtDNA. *Coptotermes formosanus* was used as outgroup

areas. They share the main component of their sex pheromones: (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (Laduguie et al., 1994, Wobst et al., 1999). But they do not hybridize, because swarming occurs generally one month apart. Behavioral tests (Clément, 1982a, e) have shown that the above molecule was generally attractive to male reproductives of the different French species, but detailed chemical analysis especially of minor compounds is needed to verify the exact pheromone composition for each species. There is currently no available data on pheromone-related species isolation mechanisms between *R. lucifugus* and the other species of the same group, i.e., *R. grassei* and *R. banyulensis*. But sympatric coexistence of these without hybridization in cities like Marseille or Bordeaux for more than 50 years suggests involvement of both long-distance and contact pheromones. The American species *R. flavipes* distinguishes itself from the European species with the main compound of its sexual pheromone being dodecyl propanoate (Clément et al., 1989).

Neotenics and workers. For hybridization to occur when colonies merge, the aggression index must be low. The interspecific aggression index (Ag) (Table 4) between *R. santonensis* and *R. grassei* is 16.5 under allopatric conditions as compared to 76.9 in winter and 34.62 in summer under sympatric conditions in the Coubre forest. Another example of high aggression index between sympatric species is given by *R. grassei* and *R. banyulensis*, i.e. 62.38 in winter and 74.82 in summer. The results of these experiments demonstrate a species isolation mechanism. Species that are geographically isolated are less aggressive towards other species, e.g., *R. santonensis* versus non-sympatric *R. grassei* (see above), *R. santonensis* versus *R. lucifugus* (Ag is 20.17 in summer), and *R. santonensis* versus *R. banyulensis* (36.27*)*. Conversely Ag is strong between *R. grassei* and *R. banyulensis*, or *R. banyulensis* and *R. lucifugus*, i.e., 78.22 in summer and 81 in winter, or *grassei* and *balkanensis*, i.e., 80.75 in winter, which are phylogenetically close and live under sympatric conditions (*grassei* and *banyulensis*).

Table 4. Intraspecific aggression (Ag) between different *Reticulitermes* species in summer (after swarming) and in winter. The confidence interval was calculated for $\alpha = 0.05$. Symbols *: sympatric populations, **: allopatric populations. 20 workers of two different species were placed in each Petri dish. 5 replicates were performed for each test. The Ag index: was calculated according to the method of Clément, 1978a. The test number is indicated in parenthesis. Ag = 0 indicates that no aggression occurred. Ag = 100 indicates that all termites were dead after 24 h

	R. santonensis	*R. sp.*	*R. balkanensis*	*R. lucifugus*	*R. l. corsicus*	*R. banyulensis*	*R. grassei*	
R. santonensis		–	–	20.17 ± 4.62 (30)	–	36.27 ± 17.07 (25)	* 34.62 ± 14.30 (50)	
R. sp.	1.75 ± 1.25 (5)		–	61.78 ± 7.76 (50)	–	–	–	
R. balkanensis	55.75 ± 29.36 (5)	57.50 ± 2.19 (5)		–	–	–		**SUMMER**
R. lucifugus	–	–	84.25 ± 10.30 (5)		57 ± 1.79 (35)	78.22 ± 9.55 (40)	74.1 ± 12.65 (55)	
R. l. corsicus	2.5 ± 2.05 (5)	67.25 ± 23.75 (5)	67.75 ± 17.03 (5)	–		–	–	
R. banyulensis	60.58 ± 9.08 (15)	66.75 ± 9.79 (5)	94.75 ± 7.37 (5)	–	81 ± 10.05 (5)		74.82 ± 11.67 (30)	
R. grassei	* 76.9 ± 5.10 (20) ** 16.56 ± 5.30 (100)	75 ± 5.31 (5)	80.75 ± 10.35 (5)	–	88 ± 3.25 (5)	62.38 ± 8.12 (45)		

WINTER

Discussion

Geographical distribution of different European species in nature

The geographical distribution of *Reticulitermes* species as determined on the basis of the data described above is presented on the map in Figure 7. Each species is genetically isolated with no evidence of hybridization. The isolation mechanisms involve swarming dates and sexual pheromones as well as aggressive behavior between workers. The large size of the sympatric zones between *R. santonensis* and *R. grassei* and between *R. grassei* and *R. banyulensis* underlines the efficacy of the species isolation.

Distribution of species in urban areas

Study of samples using chemical and molecular criteria suggests a high degree of complexity in species distribution in urban areas (Fig. 8). Urban samples show that humans have transported species far from their native range (Vieau, 1999). *R. santonensis*, the most mobile species, is found in locations as distant as Santiago (Chile) and Hamburg (Germany). In France, this species is present to the north of its natural range (Nantes, Paris, Tours…) as well as to the east and south [Basque Country (Bayonne), Toulouse and Albi (southwest France)]. A particularly intriguing finding is the presence of a sixth phenotype called *R. sp.* in dwellings in France and Italy. This phenotype, which is close to *R. balkanensis*, is so far only found in cities. *R. sp.* is clearly distinguishable from other French species based on morphological, chemical, and genetic criteria and exhibits different ecology.

Three findings deserve special attention: first, the taxonomic status of *R. santonensis* needs to be resolved. No American species has exactly the same chemotaxonomic features as *R. santonensis*. Thus, no definite explanation concerning the natural distribution of *R. santonensis* in the world can yet be given. It could either be an unknown genotype introduced from America (*R. flavipes* sub-species or sibling species) or a native European species. The only 'naturally' occurring colonies of *R. santonensis* have been found in the forests of Charente-Maritime in France.

Second, termite populations in Kent (United Kingdom) are *R. grassei* according to our morphological and chemical data. This finding has been confirmed by sequencing of the mitochondrial COI gene (Jenkins et al., pers. comm.). This suggests that *R. grassei* was introduced into the south of England from southwest France.

Third, some cities have been infested by several species. Bordeaux and Bayonne harbor *R. santonensis* and *R. grassei*. Marseille has *R. lucifugus*, *R. banyulensis*, and *R. sp.* Data from sympatric urban zones containing *R. lucifugus*, *R. banyulensis* and *R. balkanensis* have shown no indication of hybridization.

Genetic structures of colonies of each species

Molecular genetic studies provided major insight into the degree of kinship between colonies. Using this technique we can determine the genetic structure of the colony, i.e., family, tribe, or population. In a family there is only one alate

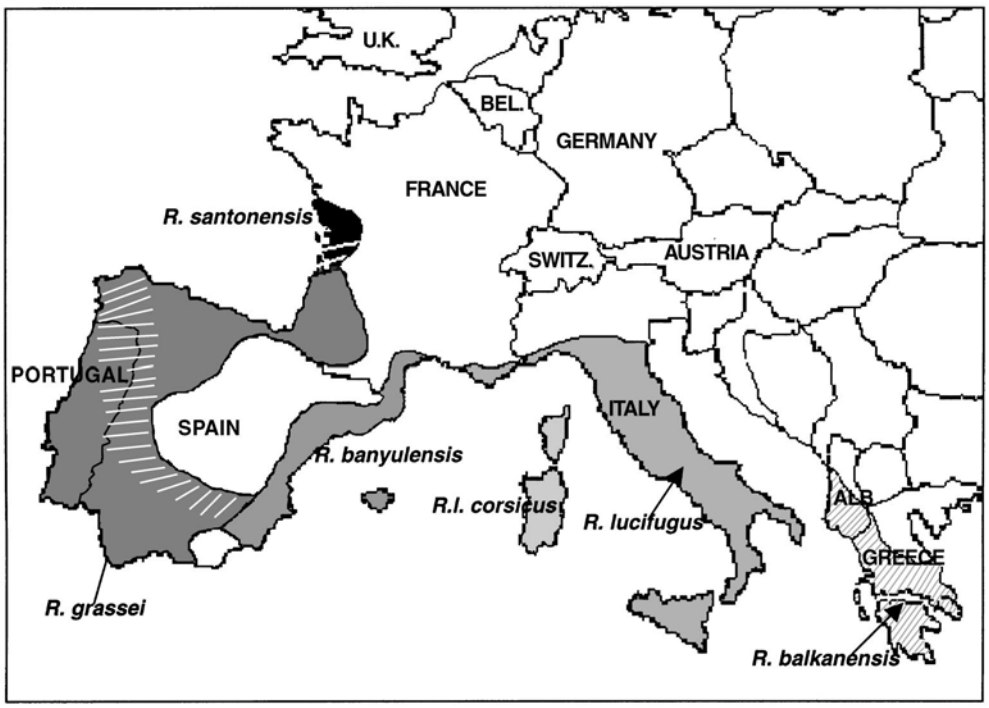

Figure 7. Natural geographical distribution of *Reticulitermes* species in Europe. Crosshatched areas indicate sympatric zones between *R. grassei* and *R. santonensis* in the north and between *R. grassei* and *R. banyulensis* in the Iberian peninsula

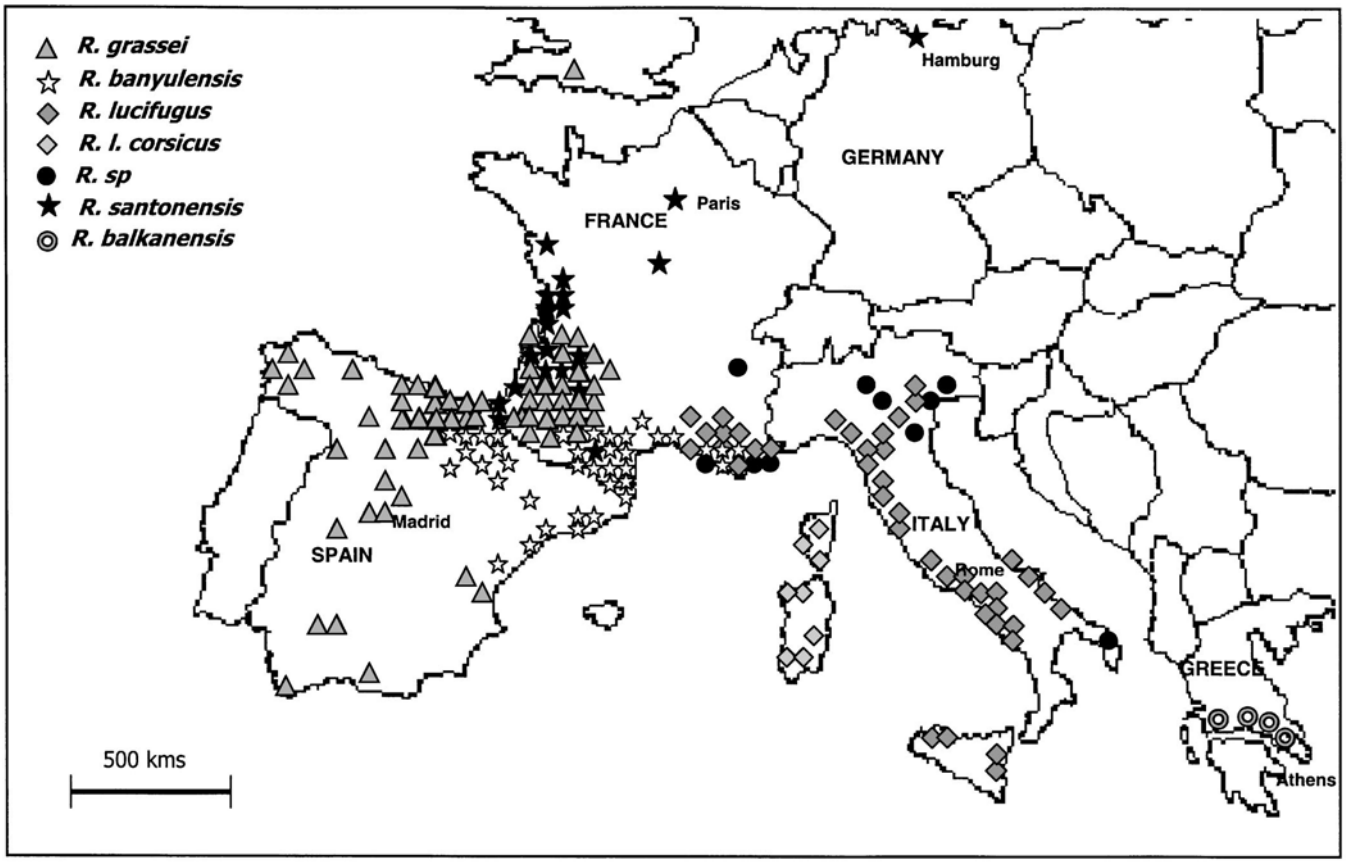

Figure 8. Urban distribution of *Reticulitermes* species in Europe

reproductive couple, the colony is closed, and descendants are brothers and sisters in accordance with Mendelian distribution of alleles. In a tribe there are many neotenic reproductives, the nest is closed, and nestmates are inbred resulting in excess homozygotes. If the colony is comparable to a population, there are many neotenic reproductives, the nest is open, and allele distribution is in accordance with the Hardy-Weinberg law. It is also possible to know if a colony is issued from reproductives from neighboring or distant colonies. Open colonies can form a vast network stretching over several kilometers (Clément, 1987a, b; Clément and Lange, 1984; Clément et al., 1988). Aggressive behavior has been evaluated between individuals from various colonies (Bagnères, 1989; Clément, 1978a, 1980, 1982f, 1987b; Clément and Bagnères, 1998). These data have been helpful, when used in conjunction with the genetic data, in understanding the relationship between colonies.

R. santonensis – 46% of colonies have a family-type structure with direct descendants from one reproductive couple. 54% of colonies consist of tribes. Similar results were reported by Vargo (2000) for *R. flavipes* and *R. virginicus* (Vargo, pers. comm.). Aggressive behavior and genetic analyses showed that societies are open.

R. grassei – In terms of genetic structure, two geographical zones can be distinguished. In southwest France, northwest Spain, and Portugal, 60% of colonies are comparable to populations, 20% are families and 20% are tribes. These colonies are genetically open. No aggressive behavior is observed between individuals in different locations in the summer, but aggression is strong during winter until the swarming period. The different colonies in the region extending from Bordeaux to Saint-Jacques de Compostelle can be considered as a single colony. The second geographical zone is the southern Iberian Peninsula in which 77% of colonies are families and only 4% can be considered as small populations. 13% are tribes. There are major genetic differences between colonies. Colonies are closed throughout the year and workers exhibit intense intraspecific aggressive activity.

R. banyulensis – 50 to 60% of colonies are families. 20 to 25% of colonies can be considered populations. 30% are tribes. There are major genetic differences between colonies. Aggressive behavior is intense between colonies resulting in closed colonies.

R. lucifugus – The genetic structure of *R. lucifugus* colonies is similar to that of *R. grassei* despite the existence of major taxonomic differences. 53% of colonies are families, 20% are populations, and 30% are tribes. Aggressive behavior between individuals from different colonies is low.

Species evolution since the last ice age

It is interesting to speculate on the evolution of sibling species in Europe since the last ice age. Two hypotheses can explain the genetic distance observed in the group including *R. grassei*, *R. banyulensis*, *R. lucifugus*, and *R. balkanensis*. Given the large distance that separates it from the other species, *R. santonensis* would not appear to be directly implicated in this recent process.

The first hypothesis involves the existence of three havens during the last ice age (Wurms era 13,000 years ago), i.e., southern Spain, Italy, and southern Balkans. As the climate warmed, each of these havens produced a species, i.e. *R. balkanensis* in Greece, *R. lucifugus* in Italy, and the *grassei-banyulensis* group, which may have diverged 8,000 years ago during the Atlantic period. Separation occurred in the south and in the central region of the Iberian Peninsula that were then too warm and dry to accommodate *Reticulitermes*. In this case the genetic similarities between *R. grassei* and *R. banyulensis* could be explained by recent speciation.

The second hypothesis involves only two havens, i.e. Greece and the southern part of the Iberian Peninsula with northward spreading of the populations and speciation of *R. grassei*, *R. banyulensis*, and *R. lucifugus* during the Atlantic period. In this context we could explain the similarities between *R. lucifugus* to the *R. banyulensis – R. grassei* clade since the separation would be more recent.

Conclusion

Although the six *Reticulitermes* species residing in both nature and urban areas of Europe are morphologically close, they present major differences in behavior, genetic structure, colony formation and resistance to pesticides (Lohou et al., 1997; Clément et al., 1999). Understanding the differences between termite species is necessary for effective termite control, especially for application of sophisticated techniques. With regard to this, it is important that the map of termite distribution is kept up to date.

A major factor in the spread of species is human intervention. Our results showed that *Reticulitermes* colonies could be found in locations as far apart as Hamburg (Germany) and Santiago and Valparaiso (Chile) and in numerous French and Italian cities north of the natural distribution area of the genus. These findings raise the likelihood of infestations of major European cities in areas where termites are not naturally present. Like Paris, Nantes, Tours, and Hamburg, many cities probably have yet undetected *Reticulitermes* colonies. The species described by Kollar (1837) as *R. flavipes* may have been another endemic European species.

It is likely that diversity and presence of numerous sibling species are a general rule in termites. The most interesting case in the USA, involves *Reticulitermes spp.* and *Zootermopsis spp*., which exhibit numerous phenotypic differences in cuticular hydrocarbons and defensive substances (Clément et al., 1986; Bagnères et al., 1990a; Haverty and Nelson, 1997; Haverty et al., 1996, 1999; Jenkins et al., 2000).

Further studies are needed to clarify several points including the presence of natural populations of *R. santonensis* in North America, the status of the different *R. flavipes* phenotypes in the United States, and the identity of *R. santonensis* and the *R. flavipes* species or phenotype. The taxonomic position of the *R. sp. nov.* phenotype, which is close but not identical *to R. balkanensis* as well as to *R. clypeatus* needs to be clarified.

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INTERSPECIFIC VARIATION IN TERPENOID COMPOSITION OF DEFENSIVE SECRETIONS OF EUROPEAN *Reticulitermes* TERMITES

ALEXANDRE QUINTANA,[1,2] JUDITH REINHARD,[1,*] ROBERT FAURE,[3] PAOLO UVA,[1,4] ANNE-GENEVIÈVE BAGNÈRES,[1,4] GEORGES MASSIOT,[2] and JEAN-LUC CLÉMENT[1,4]

[1]*CNRS, Laboratoire de Neurobiologie–UPR 9024*
31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France

[2]*Institut de Recherche Pierre Fabre*
Centre de Recherche sur les Substances Naturelles, CNRS–UMR 1973
Parc technologique du Canal, 3 Rue Ariane 31527 Ramonville, France

[3]*Université Aix-Marseille III Laboratoire de Valorisation de la Chimie Fine, CNRS–UMR 6009*
Avenue Escadrille Normandie-Niemen, 13397 Marseille Cedex 20, France

[4]*Institut de Recherche sur la Biologie de l'Insecte, UMR 6035*
CNRS–Université F. Rabelais, Faculté des Sciences et Techniques
Parc de Grandmont, 37200 Tours, France

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Abstract—Sixteen terpene compounds were isolated from the soldier defensive secretions of seven European termite taxa of the genus *Reticulitermes* (Isoptera, Rhinotermitidae). We describe species-specific mixtures of monoterpenes (α-pinene, β-pinene, limonene), sesquiterpenes (germacrene C, germacrene A, germacrene B, β-selinene, δ-selinene, γ-selinene, (E)-β-farnesene, γ-cadinene, nerolidol), diterpenes (geranyl linalool, geranyl geraniol, geranyl geranial), and one sesterterpene (geranyl farnesol). Compounds were purified by HPLC and their structures determined by means of MS spectrometry, or 1D and 2D NMR spectroscopy. Comparison of two different analytical approaches, GC-MS and HPLC with subsequent NMR spectroscopy, revealed Cope rearrangement of germacrene A, germacrene B, and germacrene C to the respective β-elemene, γ-elemene, and δ-elemene under GC conditions, thus demonstrating the limits for this analytical approach. The species-specific compound composition provides insight into taxonomy and species origin of European *Reticulitermes*.

* To whom correspondence should be addressed at current address: Research School of Biological Sciences, Visual Sciences, Australian National University, GPO Box 475, Canberra Act 2601, Australia, E-mail: reinhard@rsbs.anu.edu.au

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The biological significance of the species-specific composition of *Reticulitermes* defensive secretions is briefly discussed.

Key Words—*Reticulitermes*, Isoptera, Rhinotermitidae, subterranean termites, soldier defensive secretions, terpenes, HPLC, NMR, GC-MS, Cope rearrangement.

INTRODUCTION

Most of the 2000 known termite species have a soldier caste that is morphologically distinguished from the worker caste. Soldiers of the families Rhinotermitidae and Termitidae are equipped with a caste-specific frontal gland, the secretion of which is expelled through a pore on the top of the head (Grassé, 1982). The soldier caste usually assumes a role in defense of the termite colony, and there is abundant evidence that the frontal gland secretion is employed to chemically defend the colony against predators or competitors (e.g., Quennedey and Deligne, 1975; Prestwich, 1979a,b, 1983; Deligne et al., 1981; Grassé, 1982, 1986; Kaib, 1985). These secretions are composed of species-specific mixtures of compounds, including alkanes, ketoaldehydes, vinylketones, lactones, sesquiterpenes, cyclic diterpenes, and diterpene alcohols (reviewed in Grassé, 1984, 1986; Prestwich, 1988).

Species of *Reticulitermes* (Isoptera, Rhinotermitidae) are found throughout most of North America and Europe. The frontal gland secretions of several North American species have been analyzed, and monoterpenes (α-pinene, β-pinene, limonene, camphene, and myrcene), sesquiterpenes (γ-cadinene, cadinene aldehyde, himachalene, and bisabolene), and one diterpene alcohol (geranyl linalool) have been identified (Bagnères et al., 1990; Nelson et al., 2001). The composition of the secretion varies among taxa and geographically. In combination with cuticular hydrocarbon profiles, frontal gland secretion profiles have been used for extensive chemotaxonomic analyses of this genus in North America (e.g., Haverty et al., 1996, 1999; Haverty and Nelson, 1997; Nelson et al., 2001). Recent studies have shown that there are still numerous undescribed species of *Reticulitermes* in the United States (Jenkins et al., 2000; Nelson et al., 2001).

In Europe, the taxonomy of *Reticulitermes* has similarly been subject to years of investigation including morphological, chemical, and molecular studies (e.g., Becker, 1970; Clément, 1978, 1981, 1984; Bouillon, 1981; Bagnères et al., 1988; Clément et al., 2001). Currently, six *Reticulitermes* species and one subspecies have been described from Europe—with the accurate phylogenetic placement of newly discovered species yet to be determined. The chemistry of the defensive secretion of European *Reticulitermes* species until now has only been partially investigated. Earlier studies focused on the main compound of the secretion, geranyl linalool, which is present in all European *Reticulitermes* species and functions as an effective

deterrent and toxin against predators (Parton et al., 1981; Zalkow et al., 1981; Baker et al., 1982; Clément et al., 1988; Bagnères et al., 1990; Lemaire et al., 1990). The existence of minor compounds in the frontal gland secretions has not received much attention.

Here, we examine the chemical composition of soldier frontal gland secretions of European *Reticulitermes* species from different geographic locations, to gain further insight into relatedness, taxonomic position, and origin of each species.

METHODS AND MATERIALS

Insects. Termite colonies were collected in their natural habitat from infested logs and kept in the laboratory in containers with moist soil and wood. *Reticulitermes santonensis* and *R. grassei* were collected in Ile d'Oléron in May 2000 (Charente Maritime, France). *R. lucifugus* and *Reticulitermes*. sp. nov. were collected near Marseille (Bouches du Rhône, France) in April 2001, and *R. banyulensis* were collected near Bézier (Aude, France) in June 2000. *R. lucifugus corsicus* was collected in Corsica in June 2001, and *R. balkanensis* near Athens, Greece, in October 2000. As it is difficult to distinguish these species morphologically, verification of species identity was carried out via the hydrocarbon profiles of workers (Bagnères et al., 1988, 1990, 1991; Clément et al., 2001). Voucher specimens of the known European species were deposited at the National Museum of Natural History in Paris, France.

GC and GC-MS Analyses. Termite soldiers used for the chemical analyses were removed from colonies immediately before extraction. Five different colonies per species were investigated separately to account for colony variation. Whole body extracts of soldiers were used. Ten soldiers per colony were covered in 1 ml *n*-pentane for 2 min, the extract was removed, concentrated under nitrogen, and 2 μl were immediately injected into a GC-FID (for compound quantification) or GC-MS (for compound identification), respectively.

GC analysis was performed on a Delsi Nermag DN 200 GC, FID, with splitless injection on a fused silica capillary column (25 m, 0.25-μm film thickness, CpSil 5 WCOT, Chrompack). The temperature program ran from 38°C to 40°C at 0.5°C/min, then increased with a rate of 5°C/min to 320°C. Helium served as the carrier gas. The injector temperature was maintained at 250°C and the detector temperature at 280°C. Compounds were all quantified via GC using camphene and humulene as internal standards. Quantities were calculated as mean amount per frontal gland averaging the results from five colonies per species.

GC-MS analysis of the extracts was performed on a Hewlett Packard 5890 GC coupled to a Hewlett Packard quadrupole 5889A MS in electron impact mode

(70 eV). The samples were injected splitless on the same fused silica capillary column and with the same GC temperature program as described above. Most compounds could be identified with this approach, comparing retention time and mass spectra with those of authentic materials. Determination of the enantiomeric identity of compounds by chiral GC were not be carried out due to technical constraints.

As we dealt with whole body extracts and to ensure we only analyzed compounds that truly originated from the frontal gland, we also collected pure frontal gland secretion from individual soldiers by holding them with forceps and using a small piece of filter paper to soak up the secretion exuding from the frontal pore. The filter paper was extracted in 500 μl *n*-pentane for 2 min, the extract was taken up, concentrated under nitrogen, and 2 μl were immediately injected into the GC and analyzed as above.

NMR Analysis. Some of the compounds tentatively characterized by GC-MS proved to be unstable at high temperatures, and their structures were, therefore, likely to have been rearranged during GC-MS analysis. Several compounds also had highly similar mass spectra, and authentic references for confirmation of retention time and mass spectra were not available in all cases. To confirm MS data for those compounds, they were also analyzed by NMR spectroscopy, given that sufficient material could be obtained.

Compounds first were isolated and purified by HPLC. For each species, whole bodies of 300 soldiers from one colony were extracted in 3 ml $CHCl_3$. An aliquot of the extract (0.5 ml) was taken up, concentrated to 20 μl under nitrogen, and injected in a Hewlett Packard 1050 HPLC, with a HP 1100 Diode Array Detector from 200 to 400 nm wavelength. The column used was a RP-18 Ultrasphere ODS, 5 μm, 4.6 $\times$ 250 mm. Run duration was 60 min with an eluent mixture of 85:15 CH_3CN–H_2O at a flow rate of 0.8 ml/min in an isocratic mode. Each HPLC peak was collected as a separate fraction at the end of the column, and transferred into *n*-pentane by diphasic chemical extraction. This procedure was repeated six times until the entire sample of 3 ml was purified and fractionated. Corresponding fractions were pooled. A 2-μl sample of each HPLC fraction was analyzed by GC, as described above, to confirm the purity and to correlate HPLC and GC peaks. The isolated and purified compounds were stored at -20°C for NMR analysis.

The compound samples were transferred into NMR solvent by first completely evaporating the pentane under nitrogen, and subsequently adding 130 μl of $CDCl_3$. The samples were transferred with a glass micropipet into a capillary NMR tube for microquantity samples. The minimum sample size required for acceptable spectra with this method was in the range of a few micrograms. Microquantity NMR analyses (^{1}H, ^{13}C, COSY, HMQC, HMBC) were performed with a Bruker AVANCE 500 (500 MHz) using chloroform as internal standard.

RESULTS

By using GC-MS and 1D/2D microquantity NMR-spectroscopy, we identified species-specific mixtures of monoterpenes (α-pinene, β-pinene, limonene), sesquiterpenes (germacrene C, germacrene A, germacrene B, β-selinene, δ-selinene, γ-selinene, (E)-β-farnesene, γ-cadinene, nerolidol), diterpenes (geranyl linalool, geranyl geraniol, geranyl geranial), and a sesterterpene (geranyl farnesol) from the soldier frontal gland secretion of seven European *Reticulitermes* taxa (Table 1, Figure 1). The NMR and GC-MS spectra were consistent with literature (Table 2, Joulain and Konig, 1998), however, enantiomeric identity was not determined.

Terpenoid Identification. As confirmed by the control experiment (analysis of pure secretion of each species), all of the described compounds originate from the frontal gland secretion of the respective species and not from other body parts. The monoterpenes α-pinene (**1**), β-pinene (**2**), and limonene (**3**) were identified from *R. santonensis* using GC-MS (Figure 1, Table 2). Compounds (**4–6**) proved difficult

TABLE 1. TERPENOID COMPOSITION OF SOLDIER DEFENSIVE SECRETIONS OF EUROPEAN *Reticulitermes* TERMITE SPECIES[a]

			Quantity (μg/soldier)						
Peak	Compound[b]	R_t(min)	*R.* sp.nov.	*R.balk.*	*R.l.*	*R.l.c.*	*R.g.*	*R.b.*	*R.s.*
1	α-pinene	7.41	ND	ND	ND	ND	ND	ND	1.88
2	β-pinene	8.68	ND	ND	ND	ND	ND	ND	2.51
3	limonene	10.47	ND	ND	ND	ND	ND	ND	1.39
4	germacrene C	20.3	1.9	ND	0.4	0.08	ND	ND	ND
5	[germacrene A]	21.7	1.8	0.73	0.15	ND	ND	ND	ND
6	[germacrene B]	22.5	0.2	ND	ND	ND	ND	ND	ND
7	β-selinene	22.91	ND	ND	0.2	ND	ND	ND	ND
8	δ-selinene	23.15	ND	ND	ND	0.33	ND	ND	ND
9	γ-selinene	23.26	ND	ND	0.2	ND	ND	ND	ND
10	E-β-farnesene	23.93	0.2	0.5	ND	ND	ND	ND	ND
11	γ-cadinene	24.1	ND	ND	0.5	0.15	2.1	ND	ND
12	nerolidol	25.38	ND	ND	0.12	0.17	ND	ND	ND
13	geranyl linalool	35.02	30.0	8.53	6.28	8.13	11.22	8.23	0.37
14	geranyl geraniol	38.28	ND	ND	ND	ND	ND	ND	1.09
15	geranyl geranial	38.46	ND	ND	ND	ND	ND	ND	0.83
16	geranyl farnesol	46.19	ND	ND	ND	ND	ND	ND	46.0

[a] *R.* sp.nov.: *Reticulitermes* new species. *R.balk.*: *Reticulitermes balkanensis*. *R.l.*: *Reticulitermes lucifugus. R.l.c.*: *Reticulitermes lucifugus corsicus. R.g.*: *Reticulitermes grassei. R.b.*: *Reticulitermes banyulensis. R.s.*: *Reticulitermes santonensis.*

[b] Compounds are listed in GC peak elution order. Shown are GC retention times (R_t) in minutes and mean quantity in μg per soldier. (ND: not detected or detected at a level <0.05 μg; $N = 5$, standard deviation ranging from 1.9% to 8.2% of means). Compound names in brackets indicate identification is considered tentative.

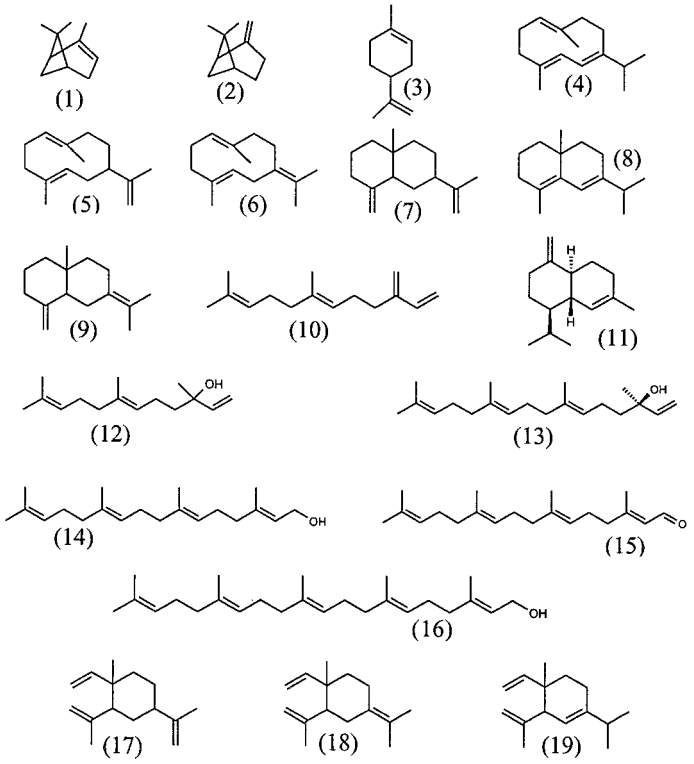

FIG. 1. Chemical structures of terpene constituents identified from European *Reticulitermes* termite defensive secretions, and structures of reference compounds (**17, 18, 19**). (**1**) α-pinene, (**2**) β-pinene, (**3**) limonene, (**4**) germacrene C, (**5**) germacrene A, (**6**) germacrene B, (**7**) β-selinene, (**8**) δ-selinene, (**9**) γ-selinene, (**10**) E-β-farnesene, (**11**) γ-cadinene, (**12**) nerolidol, (**13**) geranyl linalool, (**14**) geranyl geraniol, (**15**) geranyl geranial, (**16**) geranyl farnesol, (**17**) β-elemene, (**18**) γ-elemene, (**19**) δ-elemene.

to characterize. Their mass spectra corresponded to those of δ-elemene, β-elemene, and γ-elemene, but it is well known that elemenes can be the result of structural rearrangements under GC conditions (Cope rearrangement of germacrenes to elemenes). By using microquantity NMR spectroscopy of the isolated compound (**4**) we confirmed this compound as germacrene C (Table 2). Preliminary NMR spectroscopy of compound (**5**) showed that two of the three exomethylene signals were lacking, indicating that this compound might be germacrene A, but we did not have enough material for a full characterization. Sufficient amounts of compound (**6**) for complete NMR analysis could not be isolated either. We only obtained enough material to confirm the absence of exomethylene signals and verify the characteristic resonances of the germacrene skeleton. Therefore, the identification

TABLE 2. MASS SPECTROMETRY AND NMR SPECTROSCOPY DATA[a] FOR TERPENOID COMPOUNDS FROM EUROPEAN *Reticulitermes* TERMITE DEFENSIVE SECRETIONS AND FOR REFERENCE COMPOUNDS

Monoterpenes

α-Pinene (**1**): MS (EI, 70 eV), *m/z* (rel. int.): 136 (7), 121 (7), 105 (33), 93 (95), 91 (100), 77 (58), 67 (20), 55 (13), 43 (47), 41 (27).

β-Pinene (**2**): MS (EI, 70 eV), *m/z* (rel. int.): 136 (9), 121 (13), 105 (7), 93 (100), 91 (47), 77 (40), 69 (29), 53 (11), 41 (76).

Limonene (**3**): MS (EI, 70 eV), *m/z* (rel. int.): 136 (16), 121 (20), 107 (20), 93 (69), 91 (34), 79 (42), 69 (94), 67 (100), 53 (29), 41 (24).

Sesquiterpenes[b]

Germacrene C (**4**): ^{1}H NMR ($CDCl_3$) : δ 6.26 (1H, d, $J = 9.2$ Hz), 5.24 (1H, d, $J = 9.2$ Hz), 4.98 (1H, dd, $J = 11.4$ Hz, $J = 5.0$ Hz), 2.59 (1H, ddd, $J = 12.3$ Hz, $J = 5.6$ Hz, $J = 1.7$ Hz), 2.43 (1H, m), 2.41 (1H, m), 2.25 (1H, dt, $J = 12.0$ Hz, $J = 3.4$ Hz), 2.19 (1H, dq, $J = 12.6$ Hz, $J = 4.0$ Hz), 2.14 (1H, ddd, $J = 13.1$ Hz, $J = 4.7$ Hz, $J = 2.7$ Hz), 2.02 (1H, qd, $J = 12.6$ Hz, $J = 4.1$ Hz), 1.94 (1H, td, $J = 12.8$ Hz, $J = 4.8$ Hz), 1.77 (1H, td, $J = 12.4$ Hz, $J = 4.5$ Hz), 1.60 (3H, s, br), 1.12 (3H, d, $J = 6.8$ Hz), 1.11 (3H, d, $J = 6.8$ Hz), 1.18 (3H, d, $J = 1.2$ Hz).

[Germacrene A (**5**): complete NMR data not available, for MS-data see explanation in foot notes and text]

[Germacrene B (**6**): complete NMR data not available, for MS-data see explanation in foot notes and text]

β-Selinene (**7**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (62), 189 (47), 177 (27), 161 (50), 147 (49), 133 (58), 121 (54), 105 (100), 93 (98), 79 (72), 67 (62), 53 (36), 41 (53).

δ-Selinene (**8**): ^{1}H NMR ($CDCl_3$): δ 6.16 (1H, s, br), 2.32 (1H, hept, $J = 6.5$ Hz), 2.23 (1H, m), 2.09 (2H, t, $J = 11$ Hz), 2.06 (1H, m), 1.82 (1H, m), 1.71 (3H, s), 1.65 (1H, m), 1.51 (1H, m), 1.46 (1H, m), 1.40 (1H, t, $J = 9.3$ Hz), 1.38 (1H, m), 1.09 (3H, d, $J = 3.5$ Hz), 1.07 (3H, d, $J = 3.5$ Hz), 0.97 (3H, s). MS (EI, 70 eV), *m/z* (rel. int.): 204 (75), 189 (82), 161 (100), 147 (18), 133 (40), 119 (33), 105 (51), 93 (42), 81 (27), 69 (13), 55 (20), 41 (32).

γ-Selinene (**9**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (58), 189 (100), 177 (27), 161 (45), 147 (34), 133 (71), 119 (40), 107 (71), 93 (80), 81 (55), 69 (33), 55 (25), 41 (36).

E-β-Farnesene (**10**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (2), 161 (4), 133 (9), 120 (7), 93 (24), 69 (100), 55 (9), 41 (36).

γ-Cadinene (**11**): ^{1}H NMR ($CDCl_3$): δ 5.58 (1H, s, br), 4.69 (1H, q, $J = 1.5$ Hz), 4.58 (1H, q, $J = 1.5$ Hz), 2.41 (1H, ddd, $J = 12.8$ Hz, $J = 3.7$ Hz, $J = 3.0$ Hz), 2.22 (1H, d hept, $J = 6.9$ Hz, $J = 3.3$ Hz), 2.08 (1H, m), 2.06 (1H, m), 2.03 (1H, m), 2.02 (1H, m), 1.80 (1H, dt, $J = 12.5$ Hz, $J = 3.0$ Hz), 1.78 (1H, tm, $J = 11.5$ Hz), 1.72 (3H, s, br), 1.66 (1H, tm, $J = 10.9$ Hz), 1.51 (1H, qd, $J = 12.0$ Hz, $J = 5.8$ Hz), 1.25 (1H, tt, $J = 10.9$ Hz, $J = 3.1$ Hz), 1.15 (1H, qd, $J = 12.7$ Hz, $J = 4.1$ Hz), 0.96 (3H, d, $J = 6.9$ Hz), 0.77 (1H, d, $J = 6.9$ Hz). ^{13}C NMR δ 153.5 (C-2), 135 (C-8), 122.6 (C-7), 103.3 (C-14), 47.2 (C-5), 45.3 (C-6), 44.4 (C-1), 36.5 (C-3), 30.7 (C-9), 26.7 (C-4), 26.4 (C-11), 25.9 (C-10), 24.0 (C-15), 21.6 (C-12), 15.3 (C-13). MS (EI, 70 eV), *m/z* (rel. int.): 204 (27), 189 (7), 161 (100), 133 (24), 119 (40), 105 (54), 93 (44), 79 (33), 69 (25), 53 (18), 41 (38).

Nerolidol (**12**): ^{1}H NMR ($CDCl_3$) : δ 5.94 (1H, dd, $J = 17.3$ Hz, $J = 10.9$ Hz), 5.24 (1H, d, $J = 17.3$ Hz), 5.16 (1H, t, $J = 7.4$ Hz), 5.11 (1H, t, $J = 7.4$ Hz), 5.08 (1H, d, $J = 10.7$ Hz), 2.08 (4H, m, H-5 and H-9), 2.02 (4H, t, $J = 8.3$ Hz, H-6 and H-10), 1.71 (3H, m), 1.62 (6H, s, H-3 and H-15), 1.34 (3H, s). MS (EI, 70 eV), *m/z* (rel. int.): 204 (2), 189 (4), 161 (17), 147 (8), 136 (17), 119 (18), 107 (31), 93 (69), 81 (29), 69 (100), 55 (35), 41 (78).

TABLE 2. CONTINUED

Diterpenes

Geranyl linalool (**13**): ^{1}H NMR ($CDCl_3$) : δ 5.86 (1H, dd, $J = 17.3$ Hz, $J = 10.8$ Hz), 5.25 (1H, dd, $J = 17.3$ Hz, $J = 1.2$ Hz), 5.16 (1H, t, $J = 7.1$ Hz), 5.12 (2H, t, $J = 7.1$ Hz, H-8 and H-12), 5.08 (1H, dd, $J = 10.8$ Hz, $J = 1.2$ Hz), 2.21 (6H, t, $J = 7.4$ Hz, H-5, H-9 and H-13), 2.02 (4H, t, $J = 7.8$ Hz, H-6 and H-10), 1.71 (3H, s), 1.65 (6H, s, H-16 and H-20), 1.61 (3H, s), 1.58 (2H, t, $J = 9.6$ Hz), 1.31 (3H, s). ^{13}C NMR δ 145.2 (C-16), 135 (C-7 and C-11), 131 (C-2), 124.6 (C-4), 124.4 (C-8), 124.3 (C-12), 111.8 (C-17), 72.3 (C-15), 42.3 (C-14), 39.9 (C-6 and C-10), 28.1 (C-18), 26.9 (C-9), 25.9 (C-3), 22.9 (C-13), 17.9 (C-1), 16.2 (C-19 and C-20). MS (EI, 70 eV), *m/z* (rel. int.): 290 (1), 272 (5), 257 (2), 229 (2), 203 (6), 187 (7), 161 (11), 147 (9), 134 (18), 119 (34), 107 (36), 93 (60), 81 (54), 69 (100), 55 (29), 41 (65).

Geranyl geraniol (**14**): ^{1}H NMR ($CDCl_3$): δ 5.45 (1H, t, $J = 6.8$ Hz), 5.15 (3H, d, $J = 6.2$ Hz, H-4, H-8 and H-12), 4.18 (1H, t, $J = 6.2$ Hz), 2.01 (6H, t, $J = 7.8$ Hz, H-5, H-9 and H-13), 2.02 (6H, t, $J = 7.6$ Hz, H-6, H-10 and H-14), 1.71 (6H, s, H-1 and H-18), 1.62 (9H, s, H-3, H-19 and H-20). ^{13}C NMR δ 124.2 (C-4 and C-12), 123.5 (C-16), 59.5 (C-17), 39.8 (C-5, C-9 and C-13), 26.8 (C-6, C-10 and C-14), 25.7 (C-3), 16.2 (C-1, C-18, C-19 and C-20). MS (EI, 70 eV), *m/z* (rel. int.): 272 (7), 257 (5), 203 (5), 187 (9), 161 (13), 147 (11), 133 (18), 119 (36), 107 (29), 93 (53), 81 (55), 69 (100), 55 (27), 41 (56).

Geranyl geranial (**15**): ^{1}H NMR ($CDCl_3$): δ 10.02 (1H, d, $J = 8.1$ Hz), 5.91 (1H, d, $J = 8.1$ Hz), 5.15 (3H, t, $J = 6.9$ Hz, H-4, H-8 and H-12), 2.26 (6H, m, H-5, H-9 and H-13), 2.08 (6H, t, $J = 7.6$ Hz, H-6, H-10 and H-14), 1.71 (3H, s), 1.64 (3H, s), 1.63 (6H, s, H-19 and H-20), 1.62 (3H, s). ^{13}C NMR δ 128 (C-16), 124.2 (C-4, C-8 and C-12), 40.6 (C-5, C-9 and C-13), 26.8 (C-6, C-10 and C-14), 26 (C-1), 17 (C-3, C-18, C-19 and C-20). MS (EI, 70 eV), *m/z* (rel. int.): 288 (2), 270 (5), 252 (2), 203 (4), 185 (7), 159 (11), 145 (18), 133 (22), 119 (25), 105 (27), 93 (36), 81 (73), 69 (100), 55 (34), 41 (64).

Sesterterpenes

Geranyl farnesol (**16**): ^{1}H NMR ($CDCl_3$): δ 5.45 (1H, t, $J = 6.9$ Hz), 5.15 (4H, t, $J = 7$ Hz, H-4, H-8, H-12 and H-16), 4.18 (2H, t, $J = 5.8$ Hz), 2.08 (8H, t, $J = 6.7$ Hz, H-5, H-9, H-13 and H-17), 2.01 (8H, t, $J = 7.3$ Hz, H-6, H-10, H-14 and H-18), 1.71 (3H, s), 1.64 (12H, s, H-3, H-23, H-24 and H-25), 1.60 (3H, s). MS (EI, 70 eV), *m/z* (rel. int.): 340 (2), 203 (2), 187 (3), 161 (9), 147 (9), 135 (13), 119 (15), 107 (18), 93 (33), 81 (58), 69 (100), 55 (18), 41 (40).

Reference compounds[c]

β-Elemene (**17**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (4), 189 (27), 161 (35), 147 (49), 133 (33), 121 (47), 107 (67), 93 (96), 81 (100), 68 (69), 55 (38), 53 (32), 41 (49).

γ-Elemene (**18**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (9), 189 (14), 161 (29), 147 (13), 133 (22), 121 (100), 105 (40), 93 (69), 79 (27), 67 (25), 55 (20), 41 (36).

δ-Elemene (**19**): MS (EI, 70 eV), *m/z* (rel. int.): 204 (4), 189 (5), 161 (38), 121 (100), 105 (20), 93 (85), 77 (18), 69 (4), 55 (11), 41 (27).

[a] NMR analyses carried out to confirm MS data where required and given that sufficient material could be isolated.

[b] MS data for germacrenes A, B, and C (compounds **5**, **6**, and **4**) correspond to those of β-, γ-, and δ-elemene (compounds **17**, **18**, and **19**) (Cope rearrangement, for further explanation see text).

[c] Authentic references analyzed for Cope rearrangement verification.

of compounds (**5**) and (**6**) as germacrenes A and B is considered tentative (Figure 1, Table 2).

The sesquiterpenes β-selinene, δ-selinene, and γ-selinene (compounds **7–9**), (*E*)-β-farnesene (**10**), γ-cadinene (**11**), and nerolidol (**12**) were identified by

GC-MS. Additional NMR-analyses for some of the compounds confirmed the identification (Figure 1, Table 2). Based on GC-MS data and UV absorption at 254 nm (HPLC) due to conjugation of the double bonds, δ-selinene (**8**) was clearly distinguished from β-selinene (**7**) and γ-selinene (**9**). Importantly, this is the first report of nerolidol (**12**) from the genus *Reticulitermes*.

The diterpene geranyl linalool (**13**) was identified in all taxa by MS and NMR analysis (Figure 1, Table 2). We also identified the oxygenated diterpenes geranyl geraniol (**14**) and geranyl geranial (**15**), as well as the sesterterpene geranyl farnesol (**16**) from the defensive secretion of *R. santonensis*. This is the first report of compounds (**14–16**) from insect defensive secretions.

Interspecific Variation. The monoterpenes (**1**), (**2**), and (**3**) were only found in *R. santonensis*, in amounts of up to 2.51 μg/frontal gland, and none of the other species contained monoterpenes (Table 1). The sesquiterpenes **4**, **5**, and **6**, were each isolated from *R.* sp. nov. Germacrene A (**5**) was also found in *R. balkanensis* and *R. lucifugus*, and germacrene C (**4**) also in *R. lucifugus* and *R. lucifugus corsicus*. The germacrenes occurred in amounts ranging from 0.08 to 1.9 μg/gland (Table 1). Selinenes were restricted to the *R. lucifugus* group: δ-selinene (**8**) was found in *R. lucifugus corsicus*, while β-selinene (**7**) and γ-selinene (**9**) occurred in *R. lucifugus*. Selinenes occurred in amounts of less than 1 μg/gland (Table 1).

(E)-β-Farnesene (**10**) was common to *R. balkanensis* and the new species *R.* sp. nov. γ-Cadinene (**11**) was identified in *R. lucifugus, R. lucifugus corsicus*, and in large quantity (2.1 μg/gland) in *R. grassei* (Table 1). It was the only sesquiterpene found in *R. grassei*. Nerolidol (**12**) is present in both *R. lucifugus* and *R. lucifugus corsicus*. *R. banyulensis* did not contain any sesquiterpenes, nor did *R. santonensis*.

Geranyl linalool (**13**) was the major component in all species, with quantities ranging from 6.28 to 30.0 μg/gland, except for *R. santonensis*, where the amount averaged 0.37 μg/gland (Table 1). Instead, *R. santonensis* was the only species that contained geranyl geraniol (**14**) and geranyl geranial (**15**) in amounts of 1.09 and 0.83 μg/gland, and geranyl farnesol (**16**) as the major component with 46.0 μg/gland (Table 1).

DISCUSSION

Analysis of soldier frontal gland secretions of seven European *Reticulitermes* taxa revealed a mixture of terpenoid compounds, including monoterpenes, sesquiterpenes, diterpenes, and one sesterterpene. This finding is consistent with the fact that the defensive secretions of North American *Reticulitermes* species consist of terpenoid mixtures (Bagnères et al., 1990; Lemaire et al., 1990; Nelson et al., 2001). Terpenoid compounds are commonly found in nature, functioning as pheromones, defensive compounds, feeding deterrents, or fungicides (e.g., Juettner and Bogenschuetz, 1983; Lemaire et al., 1990; Boeve et al., 2000; Bartelt et al., 2001; Nelson et al., 2001; Shinoda et al., 2002; Wheeler et al., 2002).

Terpenoids are often difficult to characterize, requiring complementary methods for complete structure elucidation (Joulain and Konig, 1998; Bartelt et al., 2001; Hanson, 2001). GC-MS can prove insufficient for characterization of germacrenes, which under GC conditions, rearrange to the respective elemenes (Cope rearrangement) (Konig et al., 1996; De Kraker et al., 2001). Such structural rearrangements might explain differences in bioactivity sometimes encountered between natural extracts and synthetic compounds, if structure determination is based exclusively on GC-MS.

We, therefore, preferred to use HPLC at room temperature for the purification and isolation of "difficult" compounds, such as germacrenes, followed by micro-quantity NMR spectroscopy. In the case of germacrene A, this approach was insufficient, due to this compound's instability, forming four different conformations even at room temperature (De Kraker et al., 2001). Low temperature NMR spectroscopy might be the method of choice to fully characterize such compounds.

Microquantity NMR spectroscopy is a comparatively new approach, requiring only microgram amounts of a component, instead of several milligrams. This could facilitate NMR analysis of natural products from insects that often is limited due to the minute amounts available. However, considering that one might need hundreds or thousands of individuals per compound for HPLC-NMR analysis and that a large percentage of the compounds can evaporate during the transfer into NMR-solvent ($CDCl_3$), even the microquantity approach can prove insufficient, as was the case for some of the sesquiterpenes we attempted to characterize.

European *Reticulitermes* species are genetically isolated from each other with little evidence of sympatry (Figure 2) and currently no evidence of hybridization. They have been characterized based on morphological, chemical, and molecular data (Clément et al., 2001). The species-specific chemical profiles of their frontal gland secretions indicate a certain correspondence with the geographic location of the species in Europe (Figure 2).

R. santonensis from southwestern France seems to distinguish itself from the other European species, being the only species to produce the monoterpenes α-pinene, β-pinene, and limonene. It does not rely on geranyl linalool as a major component, but instead contains two other diterpenes and one sesterterpene (Table 1). Its defensive secretion shows strong chemical similarities to the secretions of the North American species *R. flavipes*, which occurs in multiple phenotypes, some of which contain the same monoterpenes as *R. santonensis*, and similarly can lack geranyl linalool (Zalkow et al., 1981; Bagnères et al., 1990; Nelson et al., 2001).

The taxonomic status of the European *R. santonensis* as a species separate from the North American *R. flavipes* has been questioned since it was described in 1924 (Feytaud, 1924). An analysis of spatial distribution and reproductive cycles suggests that *R. santonensis* is exotic to Europe, likely having been introduced from North America (Vieau, 2001). *R. flavipes* introductions into Europe are indeed

known to have happened many times since the mid-19th century (Becker, 1970, 1981). The chemical similarity of the frontal gland secretions of *R. santonensis* and *R. flavipes*, coupled with the similar hydrocarbon profiles of the two taxa, supports the hypothesis (Bagnères et al., 1990; Clément et al., 2001; Nelson et al., 2001). Further evidence is provided by recent phylogenetic analyses, which revealed a close genetic relationship between *R. flavipes* and *R. santonensis* (Clément et al., 2001; Jenkins et al., 2001). What remains to be determined is whether *R. santonensis* is, in fact, a *R. flavipes* genotype now native to France, or a population derived from another taxon from North America that is a sibling species of *R. flavipes*.

R. grassei and *R. banyulensis* extend through Spain and southern France, existing sympatrically in some areas (Figure 2). Frontal gland secretions contain only geranyl linalool and one or no sesquiterpenes, thus distinguishing them from the other species *R.* sp. nov., *R. balkanensis*, *R. lucifugus*, and *R. lucifugus corsicus*. The latter two form a distinct group displaying close chemical profiles, containing as only species selinenes and nerolidol in their secretions. *R. lucifugus corsicus* has been determined as a subspecies of *R. lucifugus*, with a *trans*-Tyrrenian distribution (Figure 2) (Clément et al., 2001; Uva et al., unpublished data).

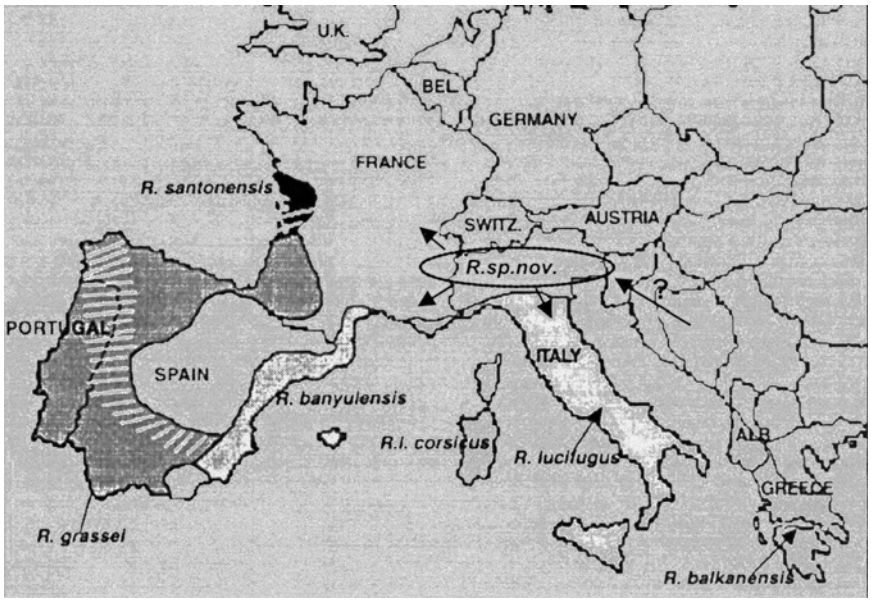

FIG. 2. Proposed endemic distribution of *Reticulitermes* species in Europe. White cross-hatched areas indicate sympatric zones between *R. grassei* and *R. santonensis* in the north and between *R. grassei* and *R. banyulensis* in the Iberian peninsula. Exact geographic distribution of the new species *R.* sp. nov. is still under investigation; arrows indicate possible origin and directions of distribution. Modified from Clément et al. (2001).

Interestingly, *R. balkanensis* from Greece shares many characteristics, such as germacrene A and especially (*E*)-β-farnesene, with the new species *R.* sp. nov. This new species is not yet fully described and has been found in urban dwellings in northern Italy and various locations in southern France (Figure 2). Our results together with other chemical, morphological, and genetic data suggest a common phylogenetic and geographic origin for *R.* sp. nov. and *R. balkanensis* (Clément et al., 2001; Uva et al., unpublished).

The biological significance of the species-specific variation in secretion profiles might lie in possible pheromonal function. There is evidence that minor compounds of the frontal gland secretion, such as monoterpenes and sesquiterpenes, play a role as alarm pheromones during colony defense, as also described for *Nasutitermes* termites (Roisin et al., 1990; Reinhard and Clément, 2002; Reinhard et al., unpublished data).

Comparative chemical analyses of *Reticulitermes* can contribute to our taxonomic knowledge of this complex genus, providing insight into species origin and evolution, and may be used as a key for species determination.

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Phylogeny of European *Reticulitermes*: a total evidence approach

Paolo UVA
Anne-Geneviève BAGNÈRES

Keywords: *Reticulitermes*, total evidence, mtDNA, cuticular hydrocarbons, termites

Introduction

En 1792, Rossi fut le premier à décrire la première espèce de termite du genre *Reticulitermes*. Des travaux réalisés dans les années 1970-1980, portant sur la morphologie (Clément, 1978, 1979), la chimie (Parton et al., 1981) et la biochimie (Clément, 1981) ont remis en cause cette classification au vu de l'existence de plusieurs groupes de populations de Reticulitermes en Europe.

Dans un premier temps, ces populations ont eu le statut de sous-espèce en absence de preuves sur leur interstérilité naturelle (Clément, 1978). Ensuite, la présence de mécanismes d'isolement reproductif (géographiques et physiologiques) a conduit à définir un certain nombre d'espèces et une sous-espèce (Clément et al., 2001) : R. santonensis dans l'ouest de la France ; R. grassei dans le sud-ouest de la France, le nord-ouest et le sud de l'Espagne et le Portugal ; R. banyulensis dans le centre et le nord-est de l'Espagne et le sud-ouest de la France ; R. lucifugus en Italie et le sud-est de la France; la sous-espèce R. l. lucifugus en Corse (France) et en Sardaigne (Italie) et R. balkanensis dans les Balkans. Les analyses des hydrocarbures cuticulaires et d'une région d'ADNmt vont dans le sens d'une synonymie

R. santonensis (Europe)-R. flavipes (USA), comme ce qui avait précédemment été proposé par Feytaud (1925). Un nouveau phénotype a également été identifié en France et dans le Nord de L'Italie (Marini and Mantovani, 2002 ; Uva et al. submit.).

Concernant à présent la partie Est du bassin méditerranéen, la taxonomie des Reticulitermes n'est pas encore bien établie : les populations sont identifiées avec l'ancienne nomenclature R. lucifugus Rossi (1792) qui est actuellement homonymique avec les populations distribuées en Italie et dans le Sud de la France. Seule l'espèce Reticulitermes présente en Israel a un statut spécifique, il s'agit de R. clypeatus (Lash, 1952). Un travail récent avec des marqueurs mitochondriaux devrait élucider les relations phylogénétiques dans cette partie de la région Paléarctique (Austin et al., in press).

Des études phylogénétiques récentes employant des marqueurs mitochondriaux (Jenkins et al., 2001 ; Marini et Mantovani, 2002) ont été déjà réalisées pour analyser les relations phylogénétiques des Reticulitermes européens. Elles présentent cependant une vision partielle de la systématique à cause d'un échantillonnage non exhaustif des taxa (absence de R. banyulensis, R. balkanensis et R. l. corsicus).

La discrimination des différentes espèces de Reticulitermes sur la base des caractères morphologiques est souvent difficile, comme l'a souligné Jenkins et al. (2000). En outre, la plasticité morphologique observée rend difficile la sélection de caractères diagnostiques. Des marqueurs biochimiques ont ainsi été développés, notamment les hydrocarbures cuticulaires et des séquences d'ADNmt.

Les Reticulitermes présentent un profil chimique d'hydrocarbures cuticulaires spécifique de l'espèce, avec des

variations quantitatives au sein d'une même espèce. Les proportions relatives d'hydrocarbures sont classiquement analysées par analyse multivariée qui permet une représentation des variableé (hydrocarbures) avec un nombre réduit de facteurs. La caractérisation des patterns d'hydrocarbures cuticulaires a confirmé la séparation des Reticulitermes européens (Bagnères et al., 1988; Clément et al., 2001). Une même approche phylogénétique a été proposée pour présenter des hypothèses d'évolution chez les termites *Odontotermes* (Kaib et al., 1991) et Reticulitermes (Jenkins et al., 2000; Page et al., 2002): l'emploi des hydrocarbures est basé sur l'hypothèse que leur composition serait une expression du génotype du taxon examiné (Takahashi et al., 2001), et qu'ils sont homologues parmi les groupes étudiés. Les relations ancêtre-descendants sont ainsi inférées sur la base des hydrocarbures cuticulaires apomorphes (*derived character state*).

Parmi les 200 séquences d'ADN déposées dans GenBank pour le genre Reticulitermes, la quasi-totalité correspond à des séquences d'ADN mitochondrial (ADNmt). Leur distribution par rapport aux espèces n'est cependant pas homogène. En outre, dans certains cas, le même gène a été séquencé dans plusieurs études mais avec une superposition des taxa échantillonnés. Il s'agit d'un problème déjà souligné par Eggleton (2001) : l'échantillonnage pour les études phylogénétiques des Isoptères ne tient souvent pas compte d'un effort conjoint au niveau international.

Un premier rapprochement des hydrocarbures cuticulaires et des séquences d'ADNmt a été réalisé par Jenkins et al. (2000) par une analyse séparée : cette approche a permis d'inférer les relations phylogénétiques entre les espèces sympatriques de Reticulitermes aux Etats-Unis. Par la suite, Clément et al. (2001) ont comparé les espèces de Reticulitermes européens à l'aide d'une approche multidisciplinaire (morphologie,

hydrocarbures cuticulaires, ADNmt), mais jusqu'à présent, aucun travail n'avait été réalisé avec une analyse combinée (*Total Evidence*) des hydrocarbures cuticulaires et des séquences d'ADNmt.

La discussion controversée concernant les analyses de "Total Evidence" versus "analyse séparée" a commencé lorsque Kluge (1989) a suggéré de toujours utiliser la totalité des caractères (=*total evidence*) dont on dispose pour optimiser le pouvoir informatif des caractères utilisés : les jeux de données (*data sets*) (e.g. gènes, morphologie et ADN) soumis à des contraintes différentes, i.e. soumis à différents taux d'évolution, peuvent interagir et augmenter le support de la topologie de l'arbre à différents niveaux. Une approche différente a été proposée par Miyamoto and Fitch (1995) qui suggèrent d'analyser les partitions de données séparément pour obtenir plusieurs estimations indépendantes des arbres : la congruence des topologies fournit un support à la structure phylogénétique obtenue (*taxonomic congruence*).

Un compromis entre *total evidence* et *taxonomic congruence*, nommé *conditional data combination*, a été proposé par Bull et al. (1993). Les partitions sont tout d'abord analysées séparément, et ensuite sont soumises à un test de homogénéité: si le test est non-significatif, les partitions sont combinées et analysées simultanément. Plusieurs méthodes ont été proposés pour tester l'homogénéité des partitions (Huelsenbeck et al., 1996).

Dans cette étude nous avons employé deux loci moléculaires (tRna-Leu et ND1) et les hydrocarbures cuticulaires. Ces données ont été utilisées pour (i) décrire les relations phylogénétiques au sein du genre Reticulitermes en Europe sur la base des variations de l'ADNmt et étudier leur congruence avec une analyse phylogénétique des hydrocarbures cuticulaires; (2) proposer une approche de *total evidence* pour

une analyse combinée ANDmt-hydrocarbures cuticulaires et évaluer les avantages-inconvénients de cette méthodologie vis à vis d'une analyse séparée.

Materials and methods

Echantillons

Tableau 1 – Liste du materiel analysé

Les échantillons ont été obtenus a partir de stock de laboratoire gardés à –20°C ou d'individus vivants. Les détails pour chaque point de récolte sont montrés dans le tableau 1. Les spécimens ont été initialement identifiés par la détermination du phénotype chimique, dans notre cas le profile des hydrocarbures cuticulaires, avant l'extraction de l'ADN. Des données ont été obtenues à partir d'études précédentes (pour les hydrocarbures cuticulaires: Bagnères et al., 1988, 1990 ; Haverty et al., 1996 ; Clément et al., 2001 ; Uva et al., submitted; pour les séquences d'ADNmt : Uva et al., submitted). Un total de 51 échantillons a été analysé. *Coptotermes formosanus* Shiraki a été choisi comme outgroup sur la base des relations avec le genre *Reticulitermes*: la sous-famille des Heterotermitinae, qui inclut le genre *Reticulitermes*, aurait évolué à partir des Coptotermitinae (Krishna, 1970).

Molecular techniques

Total DNA extraction was performed from a single termite head using a modified version of the method described by Kocher et al. (Kocher et al., 1989). Amplifications were performed with a Biometra 96T1 in a 50 µl reaction mixture with the following cycles: 2 min denaturation at 92°C followed by 35 cycles of 15 s at 92°C, 45 s at 45-55 (en fonction des espèces), 1 min 30 s at 62°C, and a 7 min final extension at 62°C for 7 min. The amplified products were sent to MWG-Biotech for the sequencing reactions after a purification with

the Qiagen-PCR purification kit. The primers for PCR amplification and sequencing were 5'-CGT TTC GAT CAT TAA AAT CTT AC-3' in 16S rRNA and 5'-ATC AAA AGG AGC TCG ATT AGT TTC-3' in NADH dehydrogenase-1 (ND1) gene. The nucleotide sequences were entered in GenBank and the accession numbers are displayed in Table 1.

Analysis of cuticular hydrocarbon

Cuticular hydrocarbons were characterized from pooled samples of 100 workers according to procedures in Bagnères et al. (Bagnères et al., 1990; Bagnères et al., 1991). Cuticular hydrocarbons were identified by gas-chromatography-mass spectrometry (GC-MS). A total of 91 cuticular hydrocarbons were used as phenotypic characters.

Phylogenetic analysis of nucleotide sequences

Sequences were aligned with CLUSTAL W (Thompson et al., 1994) using the BIOEDIT 4.8.10 sequence editor (Hall, 1999). Both parsimony and maximum likelihood were performed with the PAUP* 4.0b10 package (Swofford, 2001). Trees were drawn using TREEVIEW (Page, 1996).

With the large data set of 52 sequences we used the heuristic search algorithm with 10 random-addition replications to find the most parsimonious trees. We explored the effects of differential weighting of characters changes by using both weighted (transversion 2x over transitions) and unweighted step matrix. The level of confidence in each node was assessed with 2000 bootstrap replication (Felsenstein, 1985), as suggested by Hedges (1992). Branches having bootstrap values less than 50% were collapsed.

For the maximum likelihood analysis we used the MODELTEST program (Posada and Crandall, 1998) that uses log likelihood scores to establish the model of DNA evolution that best fits the data. We performed an heuristic search with the model selected with starting trees obtained by stepwise addition.

Branches of zero length were collapsed. Data were bootstrapped 100 times.

Phylogenetic analysis of cuticular hydrocarbons

Peak areas were converted to percent of total hydrocarbons. The proportions were coded in discrete characters: ≤ 1% = 0; 1-5% = 1; > 5% =2 and parsimony analysis were performed using the heuristic search algorithm of PAUP. Both unweighted and weighted step matrix were applied. In the weighted parsimony we assumed a higher cost for a qualitative variation (weight = 2 for the 0 ↔ 1 and 0 ↔ 2 replacements) than for a quantitative variation (weight =1 for the 1 ↔ 2 replacement). Support was assessed by bootstrap consensus trees from 2000 replications.

Phylogenetic tree comparaisons

The level of incongruence between different data sets was examined by computing the partition homogeneity test (PHT, Farris et al., 1995) implemented in PAUP. The tree topologies obtained from cuticular hydrocarbons and mDNA data sets were compared by a Templeton's test (Templeton, 1983), based on a Wilcoxon's signed-ranks test. The combined data set including 823 characters (732 molecular and 91 chemical) was analyzed using the parsimony heuristic search in PAUP, as suggested by Mishler (Mishler, 1994). Bootstrap values were calculated using 2000 bootstrap replications. To measure the contribution of each data partition to branch support in the combined analysis we used the Partitioned Bremer Support (PBS, Baker and DeSalle, 1997) using TREEROT 2 (Sorenson, 1999). The PBS is obtained by subtracting the length (in steps) of the partition on the unconstrained total evidence tree from the length of a partition on a tree constrained to have only the node of interest. If the partition supports a node in the combined analysis, the constraint tree will be longer (PBS > 0). A PBS < 0 indicates that an alternative node is

supported by the partition, so an incongruence exists between partitioned and total evidence analysis. The sum of the PBS values equals the decay index on the total evidence tree.

The consistency index (CI) (Kluge and Farris, 1969) and the retention index (RI) (Farris, 1969) were displayed for each parsimony tree. Both CI and RI range from 0 (complete homoplasy) to 1 (no homoplasy).

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Tableau 1. Liste des échantillons analysées

Tableau 2. Taille des data sets, nombre de sites *parsimony-informatives*, nombre d'arbre obtenus par maximum de parcimonie (MP), longueur des arbres, indice de consistance, indice de rétention

Tableau 3. Sites variables (ADNmt et hydrocarbures cuticulaires)

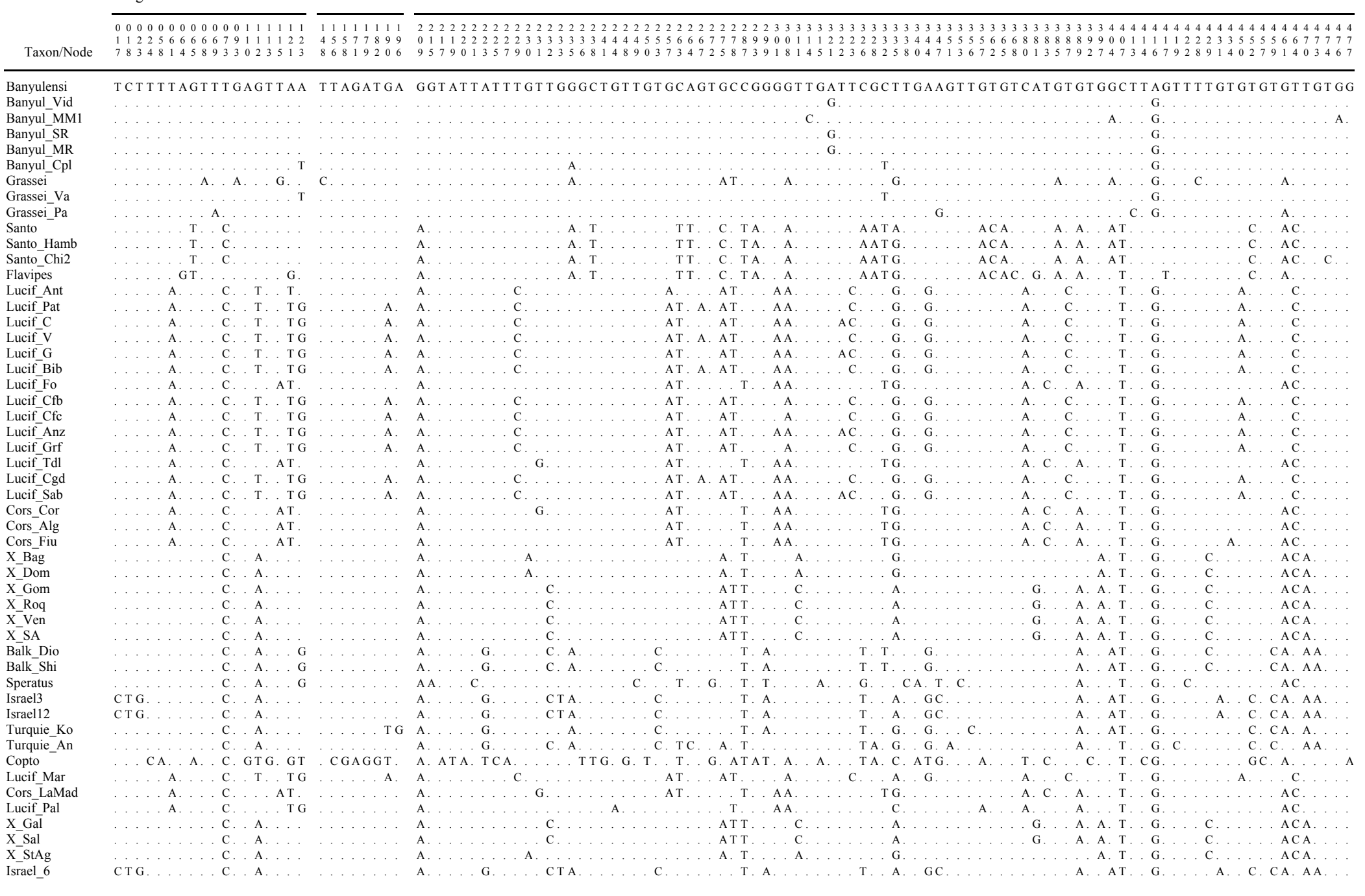
Species	Location	Code	GenBank
R. banyulensis	Beziers (34-France)	Rb-Bez	AY101825
R. banyulensis	Vidauban (83-France)	Rb-Vid	AY101826
R. banyulensis		Rb-MM1	
R. banyulensis		Rb-SR	
R. banyulensis		Rb-MR	
R. banyulensis		Rb-Cpl	
R. grassei	Forêt de la Coubre (17-France)	Rg-For	AY101827
R. grassei		Rg-Va	
R. grassei		Rg-Pa	
R. santonensis	Ile d'Oleron (17-France)	Rs-Ole	
R. santonensis	Hambourg (Germany)	Rs-Ham	
R. santonensis		Rs-Chi2	
R. flavipes	Raleigh (USA)	Rf	
R. lucifugus	Antibes (06-France)	Rl-Ant	
R. lucifugus	Patanella (Italy)	Rl-Pat	AF458614
R. lucifugus	Campo di Mare (Italy)	Rl-Cdm	AF458623
R. lucifugus	Viareggio (Italy)	Rl-Via	AF458615
R. lucifugus	Gianola (Italy)	Rl-Gia	AF458625
R. lucifugus	Bibbona (Italy)	Rl-Bib	AF458616
R. lucifugus	Follonica (Italy)	Rl-Fol	AF458621
R. lucifugus	Castelfusano-b (Italy)	Rl-Cfb	AF458618
R. lucifugus	Castelfusano-c (Italy)	Rl-Cfc	AF458619
R. lucifugus	Anzio (Italy)	Rl-Anz	AF458624
R. lucifugus	Grottaferrata (Italy)	Rl-Grf	AF458620
R. lucifugus	Torre del Lago (Italy)	Rl-Tdl	AF458622
R. lucifugus	Castelgandolfo (Italy)	Rl-Cgd	AF458617
R. lucifugus	Sabaudia (Italy)	Rl-Sab	AF458626
R. lucifugus	Martigues (13-France)	Rl-Mar	
R. lucifugus	Palermo (Italy)	Rl-Pal	
R. l. corsicus	Corsica (France)	Rlc-Cor	AY101832
R. l. corsicus	Alghero (Italy)	Rlc-Alg	
R. l. corsicus	Fiumini (Italy)	Rlc-Fiu	
R. l. corsicus	Balsey (France)	Rlc-Bal	
R. l. corsicus	La Maddalena (Italy)	Rlc-LaM	
R. sp.	Bagnacavallo (Italy)	Rsp-Bag	AY101833
R. sp.	Domène (38-France)	Rsp-Dom	AY101834
R. sp.	Château Gombert (13-France)	Rsp-Gom	AY101835
R. sp.	Roquebrun Cap Martin (06-France)	Rsp-Roq	
R. sp.	Saint Paul de Vence (06-France)	Rsp-Ven	
R. sp.	Sophia Antipolis (06-France)	Rsp-SA	AY101836
R. sp.	Galatina (Italy)	Rsp-Gal	
R. sp.	Salsomaggiore (Italy)	Rsp-Sal	
R. sp.	Sant Agata (Italy)	Rsp-StA	
R. balkanensis	Dionissos (Grece)	Rbk-Dio	AY101837
R. balkanensis	Shinias (Greece)	Rbk-Shinias	
R. speratus	Ibaraki (Japan)	Rspe	AY101838
R. clypeatus	Ben Shemen (Israel)	Rcly-3	AY101839
R. clypeatus	Ben Shemen (Israel)	Rcly-6	
R. clypeatus	Holon (Israel)	Rcly-12	
R. l.-Turkey	Konya (Turkey)	RlT-Kon	AY101840
R. l.-Turkey	Ankara (Turkey)	RlT-Ank	AY101841
C. formosanus	Baton Rouge (USA)	Cf	AY101842

Table 1

Locus	Size	PI	No. Of MPTs	TL	CI	RI
16S-rRNA	132	10	6	28	0,821	0,954
tRNA-Leu	65	1	4	9	1	1
NADH-1	535	103	400	280	0,654	0,893
mtDNA	732	114	75	320	0,672	0,898
Cuticular hydrocarbons	91	67	406	318	0,346	0,756
TE	823	181	66	695	0,468	0,803

Size: size of locus in base pairs (mtDNA) or number of cuticular hydrocarbons
PI: number of parsimony informative sites
No. Of MPTs: number of most-parsimonious trees recovered
TL: tree lenght of most-parsimonious trees
CI: ensemble consistency index (Kluge and Farris, 1969)
RI: ensemble retention index (Farris, 1989)

Table 2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tRNA-Leu
NADH-1
Taxon/Node
Banyulensi TCTTTTAGTTTGAGTTAA TTAGATGA GGTATTATTTGTTGGGCTGTTGTGCAGTGCCGGGGGTTGATTCGCTTGAAGTTGTGTCATGTGTGGCTTAGTTTTGTGTGTGTTGTGG
Banyul_Vid
Banyul_MM1
Banyul_SR
Banyul_MR
Banyul_Cpl
Grassei
Grassei_Va
Grassei_Pa
Santo
Santo_Hamb
Santo_Chi2
Flavipes
Lucif_Ant
Lucif_Pat
Lucif_C
Lucif_V
Lucif_G
Lucif_Bib
Lucif_Fo
Lucif_Cfb
Lucif_Cfc
Lucif_Anz
Lucif_Grf
Lucif_Tdl
Lucif_Cgd
Lucif_Sab
Cors_Cor
Cors_Alg
Cors_Fiu
X_Bag
X_Dom
X_Gom
X_Roq
X_Ven
X_SA
Balk_Dio
Balk_Shi
Speratus
Israel3
Israel12
Turquie_Ko
Turquie_An
Copto
Lucif_Mar
Cors_LaMad
Lucif_Pal
X_Gal
X_Sal
X_StAg
Israel_6

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4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6
7 8 8 8 8 8 8 9 0 0 0 1 1 2 2 2 2 3 3 4 4 4 5 5 5 5 5 6 6 7 7 7 7 8 9 0 0 0 1 1
9 2 3 5 6 8 9 1 0 6 9 0 5 1 4 7 8 0 3 0 3 0 3 5 1 4 5 7 0 3 2 5 6 8 4 0 0 2 8 1 4

T G T G C G A T G T T A C G G T T A A T G T T A T G T T G G G T T T A A T A A
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. . . . . .
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. . . . . C . . . . . G
. . . . T . . . . . C . . . . . A . C . . . . . G
. . . . T . . . . . A . . . . . G
. . . . T . . . . . G
. . . . T A . . . . . G . . G . . . . . A . . . . . G
. . . . T A . . . . . G . . G . . . . . A . . . . . G
. . . . T A . . . . . G . . G . . . . . A . . . . . G
. . . . T A . . . . . A . G . . . . . A . A . . . . . C . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . . . C . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . . . C . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . . . C . . . . . G
. . . . T . . C . . . . . A . . . . . A . . . C . . . . . G
. . . . . C . . . . . A . . . . . A . . . C . . . . . G
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. A C . T . . C . . . G . . . C . . . . . A . . . . . A A . . . . . G
. A C . T . . C . . . G . . . C . . . . . A . . . . . A A . . . . . G
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. . C . T . . . . . G T . . C . G . . . . . C . . . . A A . . . . . G
. . C . T . . . . . G T . . C . G . . . . . C . . . . A A . . . . . G
C . C . T . . . . . G . . . . . C . C . A A . . . . G . . . G
. . C . T . . . . C . G . . . . . G . . . . . A A . . . . . G
. . C . T . . . . C . G . . . . . G . . . . . A A . . . . . G
. . C . T . . . . . G T . . . C G . . . . C . . . . . T A . C . . . . . G
. . C . T . . . . . G . . . C . G . . . . C . . . . . A A . . C . . . . .
. . . A T . G . A . C . T . . . . . G C . C . . C T . . T . A . . A G T . G .
. . . . T . . C . . . . . A . . . . . A . A . . . . . G
. . . . T . . C . . . . . A . . . . . A . . . C . . . . . G
. . . . T . . . . . A . . . . G . . . . . C A . . . . . G
. A C . T . . C . . . G . . . C . . . . . A . . . . . A A . . . . . G
. A C . T . . C . . . G . . . C . . . . . A . . . . . A A . . . . . G
. A C . T . . C A . . G . . A C . . . . . A . . . . . A A . . . . . G
. . C . T . . . . C . G . . . . . G . . . . . A A . . . . . G
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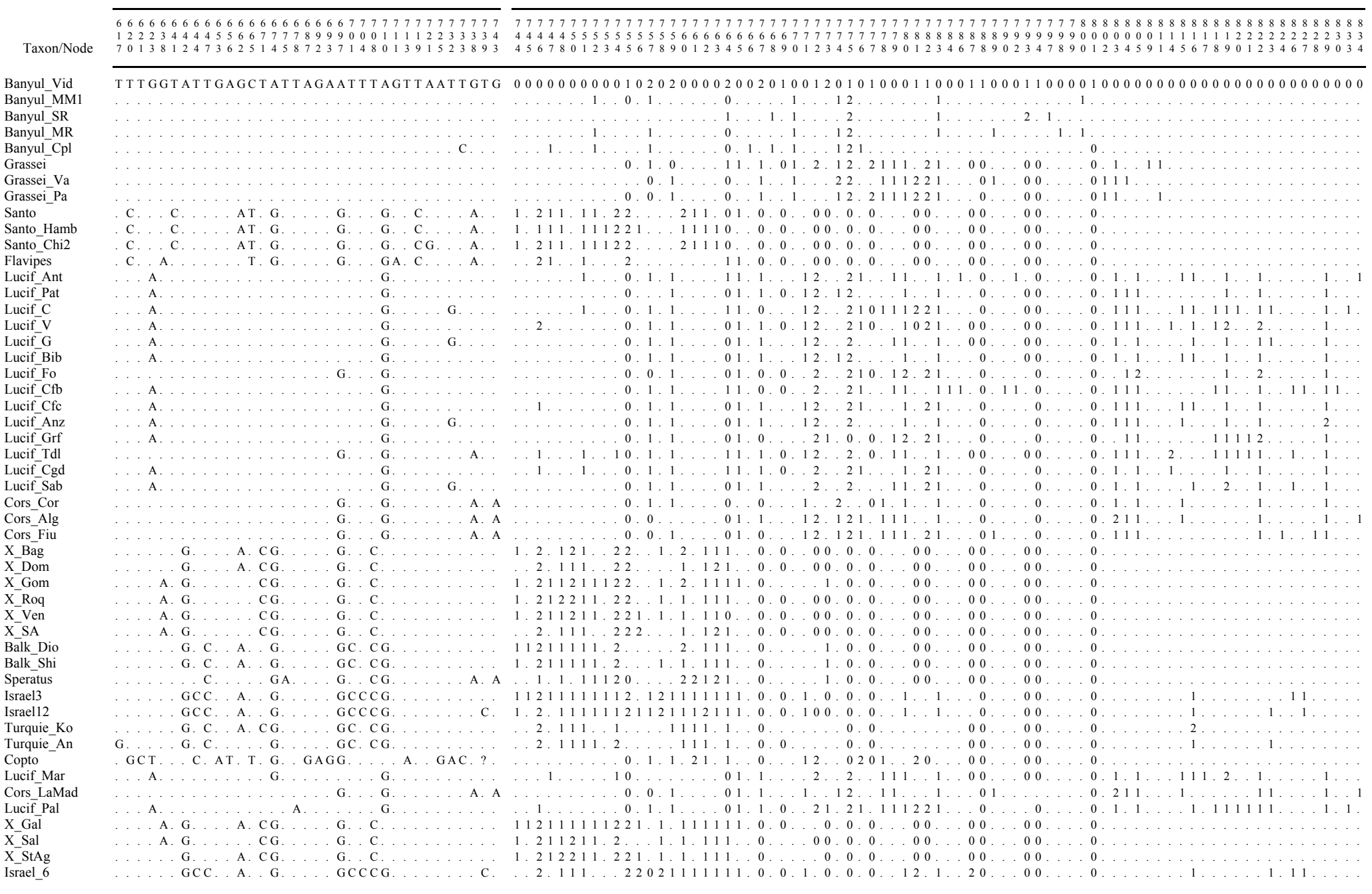
Cuticular hydrocarbons
Taxon/Node
Banyul_Vid
Banyul_MM1
Banyul_SR
Banyul_MR
Banyul_Cpl
Grassei
Grassei_Va
Grassei_Pa
Santo
Santo_Hamb
Santo_Chi2
Flavipes
Lucif_Ant
Lucif_Pat
Lucif_C
Lucif_V
Lucif_G
Lucif_Bib
Lucif_Fo
Lucif_Cfb
Lucif_Cfc
Lucif_Anz
Lucif_Grf
Lucif_Tdl
Lucif_Cgd
Lucif_Sab
Cors_Cor
Cors_Alg
Cors_Fiu
X_Bag
X_Dom
X_Gom
X_Roq
X_Ven
X_SA
Balk_Dio
Balk_Shi
Speratus
Israel3
Israel12
Turquie_Ko
Turquie_An
Copto
Lucif_Mar
Cors_LaMad
Lucif_Pal
X_Gal
X_Sal
X_StAg
Israel_6

Analyse comparative des populations italiennes de **Reticulitermes lucifugus**

La péninsule italienne constitue un modèle d'étude intéressant en raison de sa conformation géographique.

La péninsule étant développée en longueur, l'étude des colonies de termites présentes le long de ses côtes permet d'observer la présence de variations phénotypiques ou génotypiques selon un cline Nord-Sud. Ces variations seraient corrélées à un gradient climatique (Nielsen et al., 1999) et/ou à une colonisation de la péninsule à partir des régions méridionales, à la suite de la formation d'un refuge au cours des glaciations. De plus, la présence d'îles rattachées au continent dans le passé est encore plus intéressante puisqu'elle fournit un cas intéressant pour étudier l'évolution des espèces en milieu insulaire.

Outre l'étude des relations au sein des *Reticulitermes* européens, nous nous sommes aussi intéressés au cas particulier des populations italiennes de *Reticulitermes lucifugus* et de la sous-espèce *R. l. corsicus*.

L'article VI présente les résultats d'une approche multidisciplinaire comprenant des tests de comportement, l'analyse des hydrocarbures cuticulaires et le séquençage du fragment d'ADN mitochondrial ND1.

Les données comportementales et chimiques ont été récoltées au cours de mon stage de DEA. Elles ont ensuite été analysées et intégrées avec les séquences d'ADN mitochondrial.

Les résultats des tests d'agression montrent des colonies modérément agressives, et ceci indépendamment de la distance géographique intercoloniale. Un tel comportement pourrait être en relation avec le rapport coûts/bénéfices de la fusion coloniale. Ce rapport dépendrait du nombre de nymphes présentes à l'intérieur de la colonie : la fusion avec une colonie possédant de nombreuses nymphes serait désavantageuse pour la colonie receveuse car les ouvriers devraient nourrir des nymphes qui ne leur sont pas apparentées (Matsuura et Nishida, 2001).

Nous avons observé une corrélation entre la localisation géographique des colonies, la composition des hydrocarbures cuticulaires et la distribution des

haplotypes. Une distribution trans-méditerranéenne a également été observée pour la sous-espèce *Reticulitermes lucifugus corsicus*.

- **Article VI** : **Uva, P.**, J.-L. Clément and A.-G. Bagnères. Colonial and geographic variations in behaviour, cuticular hydrocarbons and mtDNA of Italian populations of *Reticulitermes lucifugus* (Isoptera: Rhinotermitidae). Accepté avec révision pour publication in *Insectes Sociaux.*

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0020-1812/04/020001-09 $ 1.50+0.20/0
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Insectes Sociaux

Research article

Colonial and geographic variations in agonistic behaviour, cuticular hydrocarbons and mtDNA of Italian populations of *Reticulitermes lucifugus* (Isoptera, Rhinotermitidae)

P. Uva, J.-L. Clément and A.-G. Bagnères*

Institut de Recherche sur la Biologie de l'Insecte, UMR CNRS 6035, Université F. Rabelais, Faculté des Sciences, Parc Grandmont, 37200 Tours, France, e-mail: bagneres@univ-tours.fr

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Summary. The subterranean termite, *Reticulitermes lucifugus* (Rossi), is found throughout Italy. The purpose of this study was to assess variations among colonies collected from the north to south of Italy. A multidisciplinary approach was used including behavioral tests, cuticular hydrocarbon analysis and partial sequencing of mitochondrial DNA. Results showed that Italian *R. lucifugus* populations were moderately aggressive and that aggressive behavior was unrelated to intercolonial geographic distance. Analysis of cuticular hydrocarbons demonstrated no qualitative variations between colonies, but quantitative differences were found according to caste and colony. Alkene content tended to decrease from north to south. Sequencing of mtDNA indicated kinship between two Tuscan populations and *R. l. corsicus*. This finding is suggestive of transtyrrenian distribution of the Corsican subspecies. Moreover, distance between haplotypes appears to be associated with intercolonial geographic distance.

Key words: Reticulitermes, subterranean termites, aggression, cuticular hydrocarbons, DNA sequences, ND1, Italy.

Introduction

The genus *Reticulitermes* includes the most common European termite species. Only one other genus with one species of a drywood termite (*Kalotermes flavicollis* (Fabr.)) appears around the Mediterranean area. The *Reticulitermes* species usually nest under the ground and expand the colony range using a gallery system. Colonies are founded by swarming and/or secondary reproductives. Although termites play an essential ecological role in the recycling of cellulose, *Reticulitermes* termites are known to be major pest of human structures throughout European countries.

European *Reticulitermes* termite species have been studied using several biochemical techniques. Chemical criteria were developed to assist species identification, which is difficult based solely on morphological characteristics (Vieau, 1999). Different phenotypes characteristic of each species with only minor intercolonial variations have been identified based on cuticular hydrocarbon profile (Howard and Blomquist, 1982; Clément et al., 1985; Bagnères et al., 1988, 1990, 1991) and soldier gland secretion composition (Parton et al., 1981; Bagnères et al., 1990; Lemaire et al., 1990; Quintana et al., 2003). Using these criteria, six phenotypes have been identified in Europe (reviewed in Clément et al., 2001): *Reticulitermes lucifugus* (Rossi) in Italy and southeastern France, *R. grassei* (Clément) in southwestern France (Provence), northwestern and southern Spain and Portugal, *R. banyulensis* (Clément) in southern France (Roussillon) and northeastern Spain, *R. balkanensis* (Clément) in the Balkans, *R. santonensis* (Feytaud) in western France, and *R. sp. nov.*, a new phenotype recently identified in Italy and southeastern France. Chemical criteria have also been widely used in biosystematics to assist morphometry in distinguishing sibling species (Carlson and Service, 1980; Bagnères et al., 1990; Haverty and Nelson, 1997; Haverty et al., 1991, 1996, 1999; Jenkins et al., 2000).

Agonistic behavioral tests have shown a correlation between aggression and cuticular hydrocarbon pattern in *Reticulitermes* termites (Howard et al., 1982; Bagnères et al., 1991), as well as in a *Macrotermes* termite (Jmhasly et al., 1998). However, hydrocarbon profiles are not the only determinants of aggression (Haverty and Thorne, 1989; Polizzi and Forschler, 1998; Su and Haverty, 1991; Thorne and Haverty, 1991). In agreement with the kinship selection theory (Hamilton, 1964), genetics probably also plays a role. In this regard behavioral tests can be useful to evaluate the

* *Author for correspondence.*

degree of relatedness between colonies having the same hydrocarbon phenotype. Furthermore, new data were provided by mtDNA analysis to infer both phylogenetic relationships (Jenkins et al., 2001; Marini and Mantovani, 2002; Austin et al., 2002) and population genetic structure (Jenkins et al., 2000). These data can also be useful for developing molecular diagnostics for identifying species.

As mentioned above, two species of *Reticulitermes* have been reported from Italy. The most abundant species is *R. lucifugus*, which is found throughout the country, especially along the western coast and in Sicily (Ghidini, 1956; Capra, 1948; Springhetti, 1965). A subspecies, *R. l. corsicus*, has been described in Sardinia and Corsica (Clément, 1978). The second termite species in Italy is *R. sp. nov.* (Clément et al., 2001; Bagnères, in prep.). It has been observed only in a few urban sites, but nothing is known of its natural distribution. Previous works on *R. lucifugus* from Italy have examined biology (Grassi and Sandias, 1893; Jucci, 1920, 1921, 1924, 1936; Ghidini, 1956; Springhetti, 1966), behavior (Springhetti and Amorelli, 1982), morphology (Lozzia, 1990), genetics (Clément, 1981; Marini and Mantovani, 2002; Luchetti et al., 2004) and chromosome structure (Fontana, 1980). However, until now, no attempt has been made to study the relationship between these different factors.

In this study we used aggressive behaviour tests, cuticular hydrocarbon analysis, and mtDNA haplotype determination to assess the variation among colonies of *R. lucifugus* collected in different sites in western Italy. Using this multidisciplinary approach, we were not only able to gain insight into species evolution, but also into the correlation between methods and their usefulness in the study of a termite species.

Methods and materials

Specimens

Workers and soldiers of *Reticulitermes lucifugus* were collected in March-April 1998 from 17 field colonies in pine forests. Colonies were located along the western coast of Italy. *R. l. corsicus,* used for comparison with *R. lucifugus* colonies, was collected in Corsica (France). Figure 1 and Table 1 provide further information on collection sites. Termites were maintained in rearing rooms at ambient conditions, in containers with moist soil and wood at the Centre National de la Recherche Scientifique (CNRS) in Marseille (France).

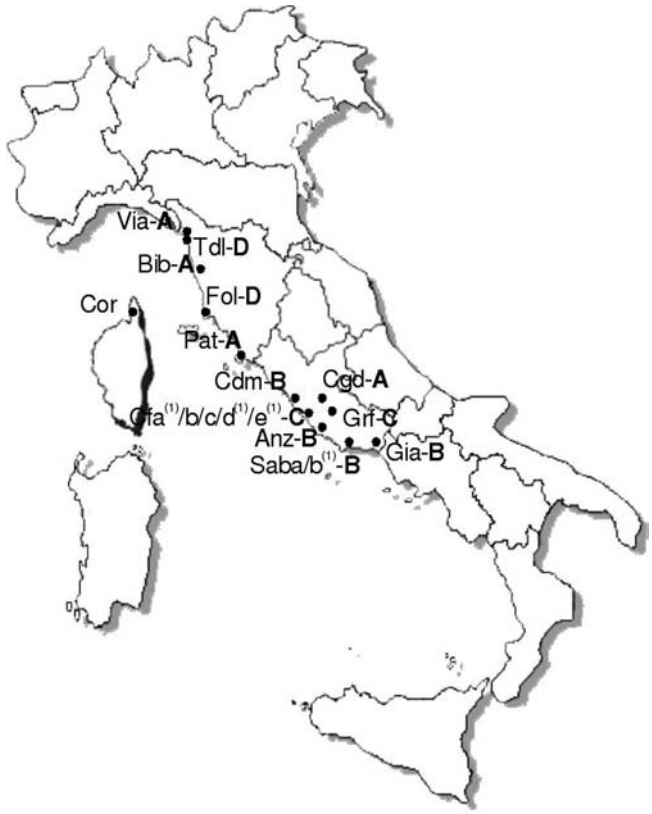

Figure 1. Map of *Reticultermes lucifugus* collection sites in Italy and Corsica. The letter code refers top the haplotypes identified in Table 4. Colonies marked with (1) were not used in mtDNA analysis

Table 1. General information for each *Reticulitermes lucifugus* collection site: distances from the northernmost colony and use in the aggression test (Ag test), cuticular hydrocarbon (C.H.) and mtDNA analysis

Location	Code	Distance (km)	Ag. test	C.H.	mtDNA
Viareggio (Lucca)	Via	0	x	x	x
Torre del lago (Lucca)	Tdl	8	x	x	x
Bibbona (Livorno)	Bib	70	x	x	x
Follonica (Grosseto)	Fol	120		x	x
Patanella (Grosseto)	Pat	180	x	x	x
Campo di mare (Rome)	Cdm	270	x	x	x
Castelfusano (Rome)	Cfa/d/e	300		x	
Castelfusano (Rome)	Cfb/c	300	x	x	x
Castelgandolfo (Rome)	Cgd	310	x	x	x
Grottaferrata (Rome)	Grf	320			x
Anzio (Rome)	Anz	340	x	x	x
Sabaudia (Latina)	Saba	380	x	x	x
Sabaudia (Latina)	Sabb	380	x	x	
Gianola (Latina)	Gia	430		x	x
Corsica	Cor				x

Agonistic behaviour

Behavioral tests were conducted during the summer of 1998 within two months after collection because some authors (Nel, 1968; Clément, 1986) observed a decrease in aggression between colonies maintained in laboratory for longer periods. All tests, except those on the Pat colony, were performed after swarming. An aggressive test between two colonies was carried out by placing 20 workers for each of the two colonies in a Petri dish, five replicates were carried out for each test. The agression index was evaluated by counting the mean number of dead ($\bar{d}$) and injured ($\bar{i}$) over the five replicusing using the formula $[\bar{d} + (\bar{i}/2)]$ (Clément, 1986). The index ranged from 0 (anagonism) to 100 (agonism). Control tests were performed using 40 workers from the same colony in five replicates also. Because of the high number of workers needed for the aggression test and the limited number of workers available, a complete pairwise comparison matrix was obtained for only 11 of the 17 colonies.

Chemical analysis

Cuticular lipids were extracted by immersing individual workers and soldiers in pentane for 10 min. Two pooled samples containing extracts from 100 workers and from 20 soldiers were used to study colonial hydrocarbon pattern. Unpooled extracts from five workers and five soldiers were used to study individual variation. Hydrocarbons were identified by gas chromatography-mass spectrometry (GC-MS) and quantified by gas chromatography (GC) as previously described (Clément et al., 2001). Relative proportions of each compound were corrected applying the FID coefficient (K = 0.042 nC + 0.11, where nC = number of Carbon atoms) (Bagnères et al., 1990). Differences between hydrocarbon patterns were estimated using the Nei index (Nei, 1972; Parton et al, 1981; Nowbahari et al., 1990).

Mitochondrial DNA

DNA extraction was performed using a modified version of the method described by Kocher et al. (1989). Termite samples were incubated for 2 h at 37°C in 100 µl of 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.1% SDS (sodium dodecyl sulfate), 50 mM dithiothreitol, and 1.5 µg of Proteinase K. After a single extraction of the incubation medium using phenol/chloroform, total DNA was precipitated with ethanol and then resuspended in 10 µl of low-concentration TE (10 mM Tris and 0.1 mM EDTA). Amplification of mtDNA was achieved by polymerase chain reaction (PCR) using primers in 16s rRNA gene (5′-CGTTTCGATCATTAAAATCTTAC-3′) and NADH dehydrogenase-1 (ND1) gene (5′-ATCAAAAGGAGCTCGATTAGTTTC-3′). This region (737 b) contains part of the ND1 gene, the t-RNA Leu and part of 16s rRNA gene. The PCR reaction mixture (50 µl/reaction) contained 37.75 µl of distilled H_2O, 5 µl of 10 X Taq DNA polymerase buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM $MgCl_2$, pH 9.0), 1 µl of dNTPs (10 mM each dNTP), 2.5 µl of 10 µM solution of each primer, 1.25 units of Taq polymerase and 1 µl of DNA template. The cycling program included an initial two-min denaturation cycle at 92°C followed by 40 cycles of 15 s at 92°C, 45 s at 50°C, 2 min at 62°C, and finally a 7-min cycle at 62°C. Amplified DNA was purified using the Quiagen-PCR purification kit and sent to MWG-Biotech for sequencing in both directions. Nucleotide sequences were entered in the GenBank database (see Table 4 for the accession numbers).

Multiple consensus sequences were aligned using BIOEDIT 4.8.10 (Hall, 1999). To display differences between haplotypes, we used a network representation by using the TCS computer program (Clément et al., 2000): it allows haplotypes to be organized into cladograms by producing an unrooted parsimony tree.

Data analysis

To test the association between matrices (aggression, geographic distances, Nei distances of hydrocarbon patterns, mtDNA sequence divergence) we used a Mantel-test, as implemented in Mantel Nonparametric Test Calculator for Windows v. 2, with 10000 permutations. Comparison of cuticular profiles was made using compounds with a relative proportion greater than 1% in at least one sample. Multivariate analysis and statistical tests were performed by using Uniwin Plus v. 3.0, Statistica v. 4.5 for Windows and R v. 1.6.1.

Results

Agonistic behaviour

The mean Ag index was higher in intercolonial tests (n = 55) than the control tests (n = 11): 21.10 versus 9.27 (Table 2). In 40 tests (72.7%) intercolonial Ag was greater than control Ag including 34 encounters involving four colonies (Cfb, Cfc, Saba and Pat). For three of these colonies, i.e., Cfb, Cfc, and Saba, Ag index was also high in control tests. Thus it is possible that higher Ag indices in these colonies could be relat-

Table 2. Aggression index (Ag) between different Italian *Reticulitermes lucifugus* colonies. Ag = 0 indicates that no aggression occured. Ag = 100 indicates that all termites were dead after 24 h. The two last columns show the mean Ag indes (mean across the 10 peir-wise tests for each colony) and the values for the control tests (40 workers from a same colony)

	Tdl	***Bib***	***Pat***	***Cdm***	***Cfb***	***Cfc***	***Cgd***	***Anz***	***Saba***	***Sabb***	Ag mean Ag	Ag mean SD	Contr.
Via	7.25	6.25	15.75	5.00	14.00	15.75	4.00	5.50	30.50	10.00	11.40	8.07	5.00
	Tdl	23.50	18.00	5.00	28.50	26.50	5.75	27.50	60.00	24.75	22.68	16.09	10.75
		Bib	39.25	5.25	20.25	20.75	3.25	4.25	38.50	7.00	16.83	13.86	5.75
			Pat	42.75	15.00	21.00	29.50	43.00	24.25	13.25	26.18	11.73	4.75
				Cdm	37.00	17.00	3.50	2.50	17.50	15.75	15.13	14.33	1.25
					Cfb	33.25	52.25	19.25	35.50	35.00	29.00	11.99	18.50
						Cfc	28.75	25.00	41.00	12.00	24.10	8.72	20.25
							Cgd	3.25	51.25	9.00	19.05	19.97	3.50
								Anz	24.00	17.75	17.20	13.33	1.50
									Saba	20.50	34.30	13.77	23.00
										Sabb	16.50	8.49	7.75

Pair-wise tests	N = 55	Mean = 21.12	SD = 14.23
Control tests	N = 11	Mean = 9.27	SD = 7.80

ed to higher intracolonial aggressive behavior. In the Pat colony Ag index was low in control tests and high in intercolonial tests. This difference may have been related to the timing of tests: Clément (1986) has observed that the aggressive response increases in winter and decreases in summer, after the swarming; unlike the other three colonies, the Pat colony contained sexual alates that had not yet swarmed. Some colonies displayed low aggression while others (e.g. Cgd and Tdl) displayed high Ag indexes. Because 100% mortality was never observed, the overall aggressivity of the colonies was classified as moderate. No correlation was found between Ag indexes and intercolonial distance (Mantel test, N.S.).

Chemical analysis

There were no qualitative differences within or among colonies in the hydrocarbon profiles of workers and soldiers from 16 colonies. A total of 58 peaks were identified including 11 *n*-alkanes (C23 to C33), 22 monomethylalkanes, 11 dimethylalkanes, 11 alkenes and 4 undetermined hydrocarbons. Monomethylalkanes were the major compounds in both workers (47%) and soldiers (45%). Identity of the hydrocarbons has been published elsewhere (Clément et al., 2001).

Quantitative analysis revealed a number of differences in the relative proportions of the HC peaks identified in workers and soldiers (Table 3). Caste differences were significant for all substances except monomethylalkanes, trienes and some unknown compounds.

A significant correlation (Spearman's rank correlation) was found between the collection site location and the proportion of cuticular alkenes (workers: $R2 = -0.62$, $df = 14$, $p < 0.05$; soldiers: $R2 = -0.63$, $df = 10$, $p < 0.05$) (Fig. 2). The proportion of alkenes in both workers and soldiers decreased from northern to central Italy. However, alkene content in termites from colonies in central and southern Italy was similar. As shown in the PCA plot (Fig. 3) carried out with all cuticular hydrocarbons, colonies located within 10 km from each other presented similar profiles, but differences did not increase with distance.

Table 3. Relative proportions of hydrocarbon classes in the *Reticulitermes lucifugus* cuticular profile

	Workers (%)	Soldiers (%)	U test[a]
Alkanes	28	26	*
Monomethylalkanes	47	45	N.s.
Dimethylalkanes	9	5	***
Monoenes	5	9	***
Dienes	5	8	**
Trienes	5	6	N.s.
Unknowns	1	1	N.s.

[a] Mann-Whitney U test *** = $p < 0{,}001$ ** = $p < 0{,}01$ * = $p < 0{,}05$ N.s. = not significant.

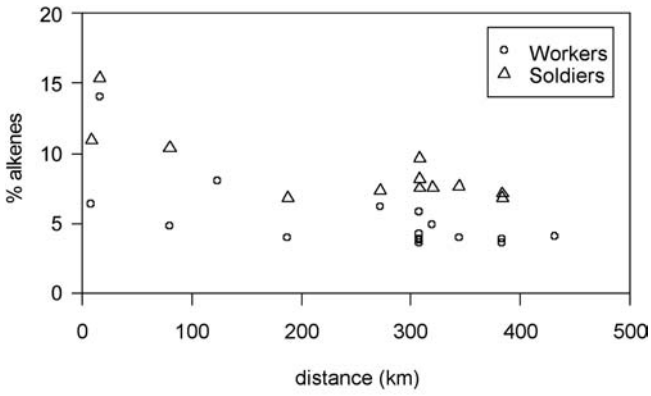

Figure 2. Plot of alkenes percentages for each colony from northern to southern Italy. The Spearmen's rank correlation coefficient between percentage and distance for workers and soldies was –0.62 ($df = 14$, $p < 0.05$) and –0.63 ($df = 10$, $p < 0.05$) respectively. The horizontal axis shows the distances computed from the northernmost colony (Via): the first colony, Via, has a distance = 0, and the last colony, Gia, is 430 km from Via

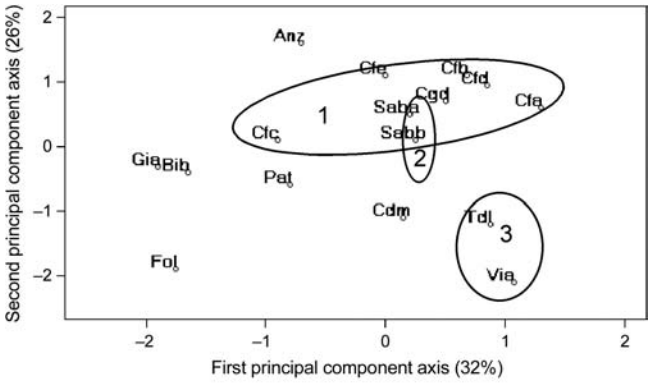

Figure 3. Colonial viration in hydrocarbon profile of *Reticultermes lucifugus* workers. The number code refers to the colonies collected in the same vicinity (intercolonial distance < 10 km). The circles surrounding each group have no statistical significance

Relationship between colony Ag index and hydrocarbon patterns

Cuticular hydrocarbon pattern did not appear to be correlated with agonistic response. No association was found between the Ag index matrix and intercolonial Nei distances (Mantel test, N.S.). A discriminant analysis was performed to select the hydrocarbons most likely to be involved in aggressive response. The Ag indexes in the pairwise comparison matrix were replaced by "n" (non aggressive) if $Ag < 20$ and "a" (aggressive) if $Ag > 30$. Using this code as the discriminant variable, each column in the matrix was analysed to select the hydrocarbons allowing clear separation between the two groups (aggressive vs. nonaggressive). Several were selected more than once including x4 (unidentified) and m23 (5-MeC27) which were selected five times and n39 (x-C29:2) which was selected three times.

Mitochondrial DNA

Four haplotypes were detected in the 27 individuals from 13 colonies (Table 4). No differences in length were observed. The aligned sequences included a total of 737 nucleotides from position 12,125 to position 12,870 in the published *Drosophila melanogaster* sequence (Clary et al., 1982). Twenty-five variable sites were detected including 3 in the 16s rRNA, 1 in the tRNA-Leu and 21 in the ND1 gene. Variation for the ND1 gene (533-bp) occurred mainly in the third-codon position: of the 21 variable characters, 6 were first-position sites, 3 were second-position sites, and 12 were third position sites. As typical for insect mtDNA (Simon et al., 1994), sequences showed a strong A/T bias (68%).

Figure 1 illustrates haplotype distribution in Italy. Although there was no clear-cut relationship with geographic areas, several trends were detected. There was an increase in the B haplotype and decrease in the A haplotype when going southward. Moreover, uncorrected sequence divergences and geographic distances between colonies appeared to be associated (Mantel test, $p < 0.05$). Uncorrected sequence divergence ranged from 0.3 to 0.5% for all haplotypes except the D haplotype (3.0–3.1%) (Table 5).

A *Reticulitermes lucifugus corsicus* sequence was added for comparison with the Italian populations. Based on comparison of p-distances, the D haplotype was close to the Corsica sample with a mean uncorrected p-distance of 1.65%. The p-distance between the Corsica sample and other colonies ranged from 4.8% to 5.0%.

Network connections produced by TCS (Fig. 4) showed two evolutionarily distinct groups: one group including the haplotypes A, B and C, and a second group including the haplotype D and the *R. l. corsicus*.

Discussion

Agonistic behaviour

The results of our behavioral tests indicate *R. lucifugus* colonies are moderately aggressive regardless of physical distance separating collection sites. By looking at Table 2, it appears that there is a considerable variation in aggression within and between colonies. Variability in aggressive behaviour between *Reticulitermes* termite species has been reported by Polizzi and Forschler (1998).

The high Ag indexes observed in intracolonial control tests could have been due to stress related to laboratory maintenance of the termite colonies. Termites maintained in a laboratory may react differently from termites living in a natural environment (Thorne and Haverty, 1991).

Real differences in aggression among individuals within and between colonies could explain the variability in behaviour observed in our tests. However, differences in the sensitivity of colonies to environmental change could also play a role. In *R. flavipes* and *R. virginicus,* Polizzi and Forschler (1998) stated that variations in aggressive behaviour were related not only to laboratory maintenance but also to experimental conditions. They suggest that chemical and behavioral cues could be responsible for stimulating aggressive behaviour in these temite species. Although controlled testing conditions simplify quantification and observation of behavior, it cannot be ruled out that aggression may be less common under natural conditions in which termites can construct barriers to keep intruders out rather than engage in combat (Springhetti and Amorelli, 1982). For these reasons, laboratory behavioral tests should be considered with caution.

An intriguing hypothesis has been proposed to account for open and closed colonies. In their experiments Matsuura

Table 4. Sequence positions of the 25 varible site in *Reticultermes lucifugus* colonies. Haplotype codes and Genbank accession numbers are showed

			16s rRNA	tRNA-Leu	ND1																			
			1	1	1	2	2	2	2	2	3	3	3	3	3	3	3	4	4	5	5	5	6	6	7
			1	1	2	2	6	7	7	8	0	2	2	3	4	8	8	8	4	6	4	7	8	2	9	3
Colony	Haplotype	GenBank	2	5	3	0	9	6	4	7	6	0	1	2	1	3	2	6	8	9	0	2	5	3	2	6	1
Via	A	AF458615	T	T	G	A	C	A	A	T	C	A	T	C	C	G	T	C	G	A	G	G	A	T	A	A	T
Bib	A	AF458616	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Pat	A	AF458614	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cgd	A	AF458617	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Anz	B	AF458624	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G
Cdm	B	AF458623	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G
Saba	B	AF458626	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G
Gia	B	AF458625	.	.	.	.	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G
Cfb	C	AF458618	.	.	.	.	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cfc	C	AF458619	.	.	.	.	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Grf	C	AF458620	.	.	.	.	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Tdl	D	AF458622	G	A	A	G	T	G	G	C	T	.	.	T	T	A	C	T	A	G	A	A	G	C	G	G	.
Fol	D	AF458621	G	A	A	G	T	G	G	C	T	.	.	T	T	A	C	T	A	G	A	A	G	C	G	G	.

Table 5. Uncorrected p-distances (%) between mtDNA haplotypes. See Table 4 for haplotype codes

	A	B	C	D	*R. l. corsicus*
A		0.407	0.271	2.985	4.645
B			0.407	3.120	4.781
C				2.985	4.645
D					1.785
R. l. corsicus					

and Nishida (2001) showed that intruders were attacked only if the nymph-to-worker ratio in their colony was higher than in the host colony. Based on this finding, these investigators suggested that behaviour was dependent on the cost-benefit relationship associated with fusion. It would be disadvantageous to merge with a colony having a high nymph-to-worker ratio because workers would have to feed unrelated nymphs. Conversely, it would be advantageous to merge with a colony having a low nymph-to-worker ratio since there would be more workers available to feed nymphs. Our observations in the Pat colony are consistent with this hypothesis as are the seasonal variations observed in *R. lucifugus* and *R. grassei* (Clément, 1986; Clément et al., 2001). In these two species, aggression is high and colonies are closed in winter when the number of nymphs in the colony is high while aggression is low and colonies are open in summer when the number of nymphs in the colony is low. This hypothesis assumes that workers are able to determine nymph-to-worker ratio from cuticular contact signals.

In our study we did not found any association between aggression and intercolonial Nei distances between hydrocarbon patterns. Since the Nei index includes all cuticular hydrocarbons, differences in one or few components could have been overlooked. The discriminant analysis selected cuticular hydrocarbons potentially implicated in aggressive behaviour. We can suppose that although the total hydrocarbon mixture is probably involved in recognition processes (Howard et al., 1982; Bagnères et al., 1991; Takahashi and Gassa, 1995), the selected hydrocarbons could be specifically implicated in aggressive response. According to the previous hypothesis, variation in these hydrocarbons could be correlated to the nymph-worker ratio in the colony and individuals may be able to detect variations in caste-specific hydrocarbon signature (Bagnères et al., 1990, 1998). An increase in the number of nymphs could lead to an increase in nymph-specific hydrocarbons in the colony (gestalt model). Testing using pure hydrocarbons and analysis to determine distribution of these hydrocarbons in different castes will be necessary to validate this hypothesis. Anyway, if caste and colony-specific mixture of hydrocarbons might be responsible for colony odor, other pheromonal or environmental components may also play a role (Shelton and Grace, 1997a, 1997b).

Chemical analysis

Saturated hydrocarbons account for 80% of the total cuticular profile of *R. lucifugus*. We observed no qualitative variation between colonies with caste and colony-specific quantitative differences. In Italy colonies collected in the same vicinity (within 10 km) showed similar profiles, but differences did not necessarily increase with distance. For example, the profiles of the Cfa, Cfb, Cfd and Cfe colonies collected in the same forest were similar not only to each other but also to those of the Anz and Tdl colonies collected in areas located 40 and 300 km away respectively. Based on these findings, we conclude that physical distance between colonies affects the cuticular pattern only for colonies located in the same vicinity. Similar results have been reported in the ant *Cataglyphis cursor* (Nowbahari et al., 1990).

A geographical trend was noted with regard to the alkene content which decreased from northern to central Italy. A similar variation has been reported concerning allele frequency in two loci of the esterase 3 system in the Italian *R. lucifugus* (Clément, 1981). The two most likely explana-

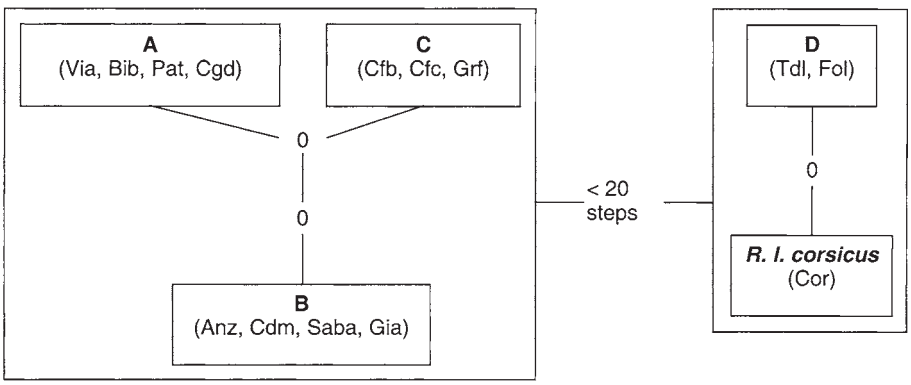

Figure 4. Haplotype network (cladogram) of mtDNA sequences for *Reticultermes lucifugus* and *R. l. corsicus* colonies. Each line in the network represents a single mutational change between haplotypes. 0 indicates a haplotype not present in the sample but inferred to be intermediate between two nearest-neighbour haplotypes. Haplotypes correspond to those given in Table 4. The network shows two evolutionarily distinct groups: A-B-C, and D-*R. l. corsicus*

tions for these intraspecific trends involve environmental conditions, e. g. temperature, food, and soil quality (Nielsen et al., 1999) and genetic variations between populations (Kimura and Ohta, 1971). In this regard Gibbs et al. (1991) showed that, although there are significant environmental effects, genetic factors are more important than environmental factors in determining variations in cuticular lipids in the grasshopper *Melanoplus sanguinipes*. However, further study will be necessary to clarify the role of hydrocarbon pattern and regulation before any definite conclusion can be drawn concerning these two hypotheses.

Mitochondrial DNA

Mitochondrial DNA has already been used to investigate intercolonial variability, phylogeography, and colonization patterns in termites (Jenkins et al., 1998, 1999, 2000, 2001; Thompson and Hebert, 1998). In our study, sequence divergences and geographical location appeared to be associated. By looking at the haplotype distribution, we observed an equivocal position for the D haplotype (Fol and Tdl colonies). It was closer to the haplotype of specimens from the *Reticulitermes lucifugus corsicus* subspecies colony collected in Corsica (uncorrected p-distance = 1.65%) than to that of specimens from other *R. lucifugus* colonies collected in Italy. Similar results have been reported by Marini and Mantovani (2002), who observed the same haplotype in *Reticulitermes* samples collected in Corsica, Sardinia and northwestern Italy. A possible explanation for this finding is that the D haplotype originated from Corsica, was transported to the continent, and hybridized with local populations. The presence of coding and noncoding regions in the mtDNA sequence studied and the absence of fossils to calibrate the molecular clock makes it difficult to estimate the time separating the two populations. The level of divergence observed in our study was less than the mean level of mtDNA divergence estimated based on a review of data in insects from the same population, different populations of the same species, and different species (Vogler et al., 1993). Average interpopulation divergence was less than 4%, and interspecific divergence including distantly related species as well as sibling species was generally greater than 5%. The distances between haplotypes (Table 5) were consistent with the hypothesis of a post-ice age recolonization from a refuge in southern Italy (Clément et al., 2001) because the A haplotype in northern Italy was closer to the C haplotype observed in central Italy than to the B haplotype observed in south central Italy.

No correlation was found between mtDNA haplotype and aggressive behavior. In a previous study using multilocus DNA fingerprinting (Husseneder et al., 1998), level of aggression was correlated with genetic distance. Beye et al., (1997) suggested that such a relation could exist if genotypes coded for compounds capable of modulating intercolonial recognition mechanisms. If these compounds are environmentally sensitive (Nielsen et al., 1999), geographically distant colonies such as those in our study would present different signals and thus the lack of correlation would be an expected finding.

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Communications scientifiques

Au cours de ces trois années de thèse, j'ai pu assister à de nombreux congrès scientifiques au niveau national et international. Il s'agit d'une opportunité extrêmement enrichissante permettant un échange dynamique avec la communauté scientifique.

À plusieurs reprises, j'ai ainsi pu présenter l'avancement de mes travaux par le biais de communications orales et affichées.

Les présentations **1 à 4** constituent en effet un résumé des résultats obtenus annuellement concernant la systématique des termites *Reticulitermes* en Europe.

Dans un premier temps, nous nous sommes intéressés plus particulièrement aux populations italiennes de *Reticulitermes lucifugus* (**1**). Par la suite, et grâce à la récolte de nouvelles données, nous avons présenté des résultats préliminaires concernant la taxonomie moléculaire des *Reticulitermes* (**2**). Enfin, un tableau exhaustif de la systématique, réalisé par une analyse comparée du comportement, de la chimie et de la génétique, a été présenté dans le cadre d'un congrès européen de l' "International Union for the Study of Social Insects" (IUSSI) (**3**). Les résultats concernant la présence d'une nouvelle espèce en Europe ont fait l'objet d'une présentation au cours du congrès international IUSSI (**4**).

Tout en gardant une approche multidisciplinaire pour la résolution des problèmes biologiques, nous nous sommes également intéressés à l'analyse comparée des données chimiques (hydrocarbures cuticulaires) et moléculaires (microsatellites). Les résultats de cette approche originale ont fait l'objet de deux présentations récentes (**5-6**), réalisées en collaboration avec les autres membres de notre groupe.

1) **1999**. **Uva, P.**, J.-L. Clément and A.-G. Bagnères. Behavioral study and variation of cuticular hydrocarbons of the Italian populations of *Reticulitermes lucifugus* (Isoptera: Rhinotermitidae). 16th Annual Meeting of the International Society of Chemical Ecology. Marseille, 13-17 novembre 1999. *Communication affichée*.

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Behavioral study and variation of cuticular hydrocarbons of the italian populations of *Reticulitermes (lucifugus) lucifugus* (Isoptera: Rhinotermitidae)

Paolo Uva, Jean-Luc Clément, Anne-Geneviève Bagnères
CNRS UPR 9024 – Laboratoire de Neurobiologie – Communication chimique
31 Ch. Joseph Aiguier 13402 Marseille Cedex 20 France
Tel: 33 4.91.16.43.72
Fax: 33 4.91.22.58.50
E-mail: uva@irlnb.cnrs-mrs.fr

Cuticular hydrocarbons were used to differentiate among sixteen italian populations of *R. (l.) lucifugus*. For each population a series of analysis was conducted. The cuticular hydrocarbons were previously identified using gas chromatography electron impact-mass spectrometry. Quantitative analysis were performed on samples of soldiers and workers using flame ionization gas chromatography. All populations contained the same cuticular hydrocarbons, but showed differences in percentage composition. This was sufficient to enable cluster and multivariate analysis (analysis of principal components) to separate the populations: a geographic variation of alkenes and dimetil-alkanes percentages has been observed. Soldiers and workers were separated by means of quantitative variations in insaturated and saturated hydrocarbons. Workers from eleven different colonies were also used to perform behaviorial tests: the test, measuring aggression between colonies, provided information about the degree of colony opening. The results showed that the italian populations were on average open and that the variation of intercolonial aggression was indipendent of geographic distance. A correlation between agonistic behavior and cuticular hydrocarbons pattern has been investigated by discriminant analysis and classification test: some components resulted to be related with aggressive reaction.

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TAXONOMIE MOLÉCULAIRE DES TERMITES *RETICULITERMES* (ISOPTERA : RHINOTERMITIDAE)

Uva P.[(1)], J. Aubert[(2)], J.L. Clément[(1)] & A.G. Bagnères[(1)]

[(1)]CNRS, Laboratoire de neurobiologie, 31 ch. J. Aiguier 13009 Marseille, France
[(2)]Université de Provence, Laboratoire de systématique évolutive, 3 pl. Victor-Hugo, 13331 Marseille, France

Le genre *Reticulitermes* (Isoptera : Rhinotermitidae) est représenté par environ 80 espèces de termites souterrains qui sont distribuées dans les régions Nearctique et Palearctique (Amérique du Nord, Asie et pourtour méditerranéen).
Les échantillons ici analysés ont été récoltés dans le Sud de l'Europe et en Amérique du Nord. L'ADN a été extrait pour chaque termite individuellement pour tenir compte du polymorphisme individuel (Jenkins et al., 1998). Les relations phylogénétiques ont été étudiées par le séquençage d'un segment de l'ADN mitochondrial (475pb du locus ND1). Les analyses phylogénétiques ont été réalisées avec PHYLIP 3.5 ; pour la construction des arbres nous avons employé à la fois l'analyse de parcimonie et les matrices de distances.
Les relations phylogénétiques dérivées des données moléculaires seront comparées aux résultats obtenus par l'étude des allozymes (Clément, 1981) et des hydrocarbures cuticulaires (Bagnères et al., 1988). Les implications biogéographiques de ces relations seront aussi discutées.

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Speciation, taxonomy and distribution of European *Reticulitermes* termites based on morphological, chemical and molecular data

Uva, Paolo[1], Bagnères, Anne-Geneviève[1], Wilfert, Lena[2], Quintana, Alexandre[1], Reinhard, Judith[1], Dronnet, Stéphanie[1], Clément, Jean-Luc[1]

[1]Laboratoire de Neurobiologie, Centre National de la Recherche Scientifique, UPR 9024, Communication Chimique, 31 chemin Joseph Aiguier, 13402 Marseille, France
Tel: (33) 491 164 372, Fax: (33) 491 225 850, E-Mail: uva@lnb.cnrs-mrs.fr

[2]Department of Animal Physiology, University of Bayreuth 95440, Bayreuth, Germany

Termites are found in temperate climates throughout the world. In Europe *Kalotermes* and *Reticulitermes* are the only two naturally residing termite genera. *Kalotermes* is represented by a single species, namely: *K. flavicollis*, a dry-wood termite living along the entire Mediterranean coastline. Six phenotypes of *Reticulitermes* have been identified on the basis of morphological, chemical (cuticular hydrocarbons, soldier defensive secretions, see also posters by Quintana et al. et Reinhard et al.) and molecular (enzymatic alleles and mitochondrial ND1 sequence) features. They are *R. santonensis* in western France, *R. grassei* in southwestern France, northwestern and southern Spain and Portugal*, R. banyulensis* in northeastern Spain, central area of the Iberian Peninsula and southwestern France, *R. lucifugus* in Italy and southeastern France, *R. balkanensis* in the Balkans and *R. sp,* a recently identified urban phenotype resembling *R. balkanensis*, in northern Italy and southeastern France. *R. santonensis* is closely kin to the American species *R. flavipes. R. grassei*, *R. banyulensis and R. lucifugus* belong to the same species complex. *R. balkanensis* and the new phenotype *R. sp.* are close to *R. santonensis* with respect to cuticular hydrocarbons, to the *lucifugus* complex with respect to DNA and to *R. clypeatus* from Israel with respect to morphology. The species status of these genotypes has been confirmed by studying the mechanisms of species isolation. Prevention of hybridization depends on the method of colony formation in each species. In *Reticulitermes* new colonies can be founded by swarming of winged sexual alates from the parent colony (swarming) or by secondary neotenic reproductives that develop among workers that have been cut off from the parent colony. Swarming period and differences in pheromones prevent hybridization by sexual alates. Interspecific aggression between workers prevents hybridization by neotenics. Based on the presented data, the taxonomy and the speciation of the *Reticulitermes* genus in Europe are discussed (Clément et al., 2001, in press).

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Genetic tools for the understanding of termite spreading in urban areas: the case of a new *Reticulitermes* species in Europe

P. Uva, S. Dronnet, M. Kutnik, J.-L. Clément and A.-G. Bagnères
Institut de Recherche sur la Biologie de l'Insecte, C.N.R.S.
Université F. Rabelais, UMR6035, 37200 Tours, France – E-mail: paolo.uva@etu.univ-tours.fr

In Europe *Reticulitermes* termites reside naturally in the Mediterranean area. However, termite damages are increasing seriously even further north than the natural limit.

Taxonomic studies based on chemical and molecular features have improved the understanding of species variations, allowing a more suitable control which need to be species-specific.

Reticulitermes santonensis is found in buildings and trees in more than half of the Paris district (France). The understanding of how colonies and populations are organized by using molecular markers (microsatellite loci) is useful to control these pests in a targeted manner.

R. grassei, a termite species occurring naturally in Southwest France and Iberian peninsula, exhibits a complex behavioral pattern that makes this species hard to eradicate.

Moreover, a new termite phenotype, named *R.sp.*, was recently found in France and Italy. We investigated the inter- and intraspecific variability of *R. sp.* using mitochondrial DNA sequences. Different regions of mtDNA change at different rates, so we used two regions: ND1 gene, a conserved marker, was used for phylogenetic inference within the *Reticulitermes* genus to identify the most likely source of this new phenotype; the A-T rich region, a highly variable marker, was used to describe the relationships existing between different populations in order to describe the potential scenario for the phenotype spreading in Europe. The data suggested a likely origin of *R.sp.* from termite populations of S/E Europe.

Finally, termite taxonomy in the Mediterranean area needs to be revised.

Keywords: TERMITES, *RETICULITERMES*, MOLECULAR DATA

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VARIATION IN CUTICULAR HYDROCARBONS AND GENETIC RELATEDNESS OF PARISIAN COLONIES OF THE TERMITE *RETICULITERMES SANTONENSIS*

Dronnet, Stéphanie; Christides, Jean-Philippe; Uva, Paolo; Vargo, Edward L; Ohresser, Marc; Kutnik, Magdalena; Clément, Jean-Luc; Bagnères, Anne-Geneviève

Institut de Recherche sur la Biologie de l'Insecte, CNRS UMR 6035, Faculté des Sciences et Techniques, Parc Grandmont, 37200 Tours, FRANCE
email: dronnet@univ-tours.fr, bagneres@univ-tours.fr

In a previous study [1], we used a technique of molecular genotyping (microsatellite markers) that gave some first informations of how colonies and populations of the subterranean termite *R. santonensis* were organized in Paris. In the present analysis, twenty workers from five different sampling points were genotyped at five microsatellite loci originally developed for *R. flavipes* [2]. We could determine relatedness among individuals, and attribute the sampling points as same or different colonies. Such assignments are also possible using cuticular hydrocarbons. Thus we determined the individual chemical signature of twenty other workers from the same colonies used in genetic analyses. All the five investigated colonies contained the same cuticular hydrocarbons that were species characteristic [3], but showed quantitative variation in percentage composition between individuals. From these preliminary data, we tried to correlate the genetic distances of colonies and the cuticular hydrocarbon patterns.

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UTILISATION DE MARQUEURS CHIMIQUES ET MOLÉCULAIRES : L'ÉTUDE DES COLONIES PARISIENNES DU TERMITE *RETICULITERMES SANTONENSIS*

Stéphanie DRONNET, Paolo UVA, Jean-Philippe CHRISTIDES et Anne-Geneviève BAGNÈRES

Institut de Recherche sur la Biologie de l'Insecte, CNRS UMR 6035, Université de Tours, Faculté des Sciences et Techniques, Parc de Grandmont, 37200 Tours, France.
E-mail :bagneres@univ-tours.fr, dronnet@univ-tours.fr

Chez les termites *Reticulitermes*, les hydrocarbures cuticulaires sont connus pour être de bons marqueurs permettant l'identification des espèces, et également l'analyse intraspécifique des variations inter-coloniales (Clément et al., 2001). L'étude de ces insectes, dont le mode de vie est souterrain (vie cryptique, réseaux de galeries), présente donc des difficultés majeures, notamment en milieu urbain.

Ces dernières années, l'utilisation croissante de marqueurs moléculaires hautement polymorphes (VNTR, DNA fingerprinting, …) a permis d'analyser de manière plus approfondie l'organisation sociale et la structuration génétique des populations.

Dans cette étude sur *Reticulitermes santonensis*, espèce présente à Paris, nous proposons une analyse consensus basée sur les profils d'hydrocarbures cuticulaires et des marqueurs microsatellites (Vargo, 2000) pour appréhender cette recherche à deux niveaux :

- intra-colonial :
  1) discrimination des castes par analyse multivariée (ACP) des proportions relatives d'hydrocarbures cuticulaires
  2) inférence de la structure reproductive basée sur l'analyse microsatellites des génotypes d'ouvriers en milieu urbain (*F*-statistics, relatedness ; Dronnet et al., 2002)

- inter-colonial : comparaison des données chimiques et génétiques basée sur une représentation graphique issue d'une approche phylogénétique (distances, PAUP).

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Conclusion générale

Le travail présenté dans ce manuscrit concerne l'étude d'un nouveau phénotype de termites du genre *Reticulitermes*, *R. sp. nov.*, identifié en France en 1998. Le but était d'identifier son aire d'origine et de définir sa position systématique dans un contexte évolutif vis à vis des autres *Reticulitermes*.

Depuis la description du premier *Reticulitermes* européen en 1792 (*Termes lucifugum* Rossi), la taxonomie de ce genre a longtemps été l'objet de controverses en raison d'une forte similarité morphologique entre les différentes populations. Ce ne sera que deux siècles plus tard qu'on aboutira à la définition de plusieurs entités taxonomiques distinctes (Clément, 1978).

L'identification d'un nouveau phénotype de termite grâce à l'analyse des hydrocarbures cuticulaires (composés majoritaires de la cuticule des arthropodes, Lockey, 1988) et de la morphologie (post-clypeus) avait soulevé la question de son identité taxonomique. L'ampleur des différences observées, par rapport aux autres *Reticulitermes* européens, ne pouvait être due à une plasticité phénotypique. Le caractère isolé de l'infestation laissait également supposer une origine liée à l'activité humaine. La présence d'une nouvelle espèce avait ainsi été envisagée.

Dans une première partie de notre travail, pour résoudre le problème de la position systématique de *R. sp. nov.*, nous avons adopté une approche comparative basée sur l'analyse des composés cuticulaires et des substances de défense de la glande frontale des soldats. Une approche phylogénétique a également été employée sur la base de séquences d'ADN mitochondrial et nucléaire. D'après 1) les résultats obtenus, 2) la présence de cette espèce dans un site naturel dans le sud de la France et 3) l'identification de cette même espèce dans de nombreux autres sites urbains (France méridionale et Italie septentrionale), il semble peu probable que *R. sp. nov.* soit arrivée en Europe à partir d'une aire extra-européenne. Comme cela a déjà été observé chez d'autres espèces en Europe (recensées par De Jong, 1998, et Taberlet et al., 1998), la distribution actuelle de *R. sp. nov.* pourrait être le résultat d'une évolution à partir de populations issues du refuge est-européen, suivi d'une migration vers le Nord le long des routes de colonisation post-glaciaires. Les populations

actuelles présentes en milieu urbain auraient été ainsi introduites à partir des régions environnantes. Depuis la première identification, d'autres sites ont été découverts à ce jour. Si nos conclusions sont correctes, leur nombre dans le sud de la France, notamment en milieu naturel, ne devrait pas cesser d'augmenter dans les années à venir.

D'autre part, le statut spécifique de *R. sp. nov.* a été validé : les populations sont phénotypiquement et génotypiquement distinctes. En même temps, des barrières contribuant à l'isolement reproductif des populations ont été observées, principalement le comportement d'agression interspécifique. Ces populations ont été ainsi attribuées au nouveau taxon ***Reticulitermes urbis***.

Dans la deuxième partie du travail, nous nous sommes intéressés aux différentes espèces de *Reticulitermes* en Europe. La pression évolutive qui est à la base de la spéciation engendre une diversité phénotypique qui peut être employée comme un outil diagnostique pour identifier les espèces. Une caractérisation des types morphologiques ou chimiques chez les *Reticulitermes* peut ainsi fournir une clef d'identification univoque. Retenons que les variations phénotypiques que l'on observe sont également influencées par des facteurs externes : un phénotype est donc le résultat de l'effet des deux composantes génétique et environnementale. Nous avons donc adopté une approche basée sur l'utilisation conjointe de plusieurs caractères indépendants dans le but de réduire les problèmes liés à l'homoplasie (e.g. convergence des caractères).

Nous avons ainsi pu caractériser les différentes espèces de *Reticulitermes* européennes, avec une attention particulière au niveau intraspécifique pour les populations italiennes de *Reticulitermes lucifugus*. Après une longue controverse sur la position systématique des *Reticulitermes* en Europe, j'espère avoir contribué à une meilleure connaissance du genre.

Le principal apport de ce travail a été de montrer la validité d'une approche multidisciplinaire. L'utilisation de séquences d'ADN soumises à différentes contraintes évolutives s'est révélée nécessaire pour retracer le passé évolutif des espèces. En plus de fournir des connaissances de base sur la vie des organismes, l'emploi de marqueurs chimiques (hydrocarbures cuticulaires et substances de défense) nous a

permis de mettre au point des outils de discrimination au niveau spécifique. Il faut également considérer que les termites sont des espèces nuisibles en milieu urbain avec un important impact économique. Une identification correcte des espèces est donc nécessaire pour appliquer une lutte efficace et ciblée.

Enfin, nous avons proposé une méthode d'analyse basée sur la combinaison de plusieurs caractères indépendants. Une telle approche peut se révéler intéressante pour la compréhension des facteurs à la base des variations phénotypiques. Dans notre cas, l'analyse conjointe hydrocarbures cuticulaires / séquences d'ADN peut fournir des informations sur l'évolution de la signature chimique mais également sur le rôle de l'environnement dans la formation des phénotypes observés.

… et j'espère également avoir fourni les éléments pour répondre à la question qui m'avait était posée il y a trois ans :

"Est-ce vraiment nécessaire de partir pendant si longtemps et si loin pour étudier des organismes aussi petits ?".

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"Considerate la vostra semenza:
fatti non foste a viver come bruti
ma per seguir virtute e canoscenza"

"Considérez votre semence:
vous ne fûtes pas faits pour vivre comme des bêtes
mais pour suivre vertu et connaissance"

Dante Alighieri
Divina Commedia
Inferno, Canto XXVI

Résumé

En Europe les termites du genre *Reticulitermes* (Isoptera : Rhinotermitidae) sont distribués dans la zone méditerranéenne, mais leur présence en milieu urbain est en cours d'expansion vers le Nord, et en dehors de leur aire de répartition naturelle.

Récemment un nouveau phénotype, *R. sp. nov.*, a été identifié en milieu urbain en France et dans le Nord de l'Italie. La distribution ponctuelle des points de récolte et sa rareté en milieu naturel avaient suggéré une importation liée à l'activité humaine, comme cela est le cas pour de nombreuses espèces invasives. L'objectif de ce travail était de définir sa position systématique au sein du genre *Reticulitermes* et d'identifier son origine géographique.

Dans un premier temps, nous avons étudié les composés présents sur la cuticule des différentes espèces de termites *Reticulitermes* européens par chromatographie en phase gazeuse (GC) et GC couplée à la spectrométrie de masse. Nous avons montré l'existence d'un pattern original d'hydrocarbures pour ce phénotype, différent des autres espèces connues dans son aire de répartition, ainsi que de fortes similarités chimique et morphologique avec les populations de la région Est-méditerranéenne (Grèce, Israël, Turquie).

Nous avons également employé une approche moléculaire (séquençage de segments d'ADN mitochondrial et nucléaire) pour définir des relations phylogénétiques au sein du genre *Reticulitermes*, ce qui a permis de confirmer l'originalité du phénotype.

Les données moléculaires obtenues, en accord avec les données chimiques, à la fois par analyse séparée et combinée (évidence totale), suggèrent une origine des populations de *R. sp. nov.* à partir d'un refuge balkanique en suivant des routes de colonisation postglaciaires. Ceci nous permet donc d'élever ces populations à un statut spécifique, en proposant pour cette nouvelle espèce le nom de *Reticulitermes urbis*.

Mots-clés : phylogénie moléculaire, hydrocarbures cuticulaires, ADN mitochondrial, *Total Evidence*, termites, *Reticulitermes*